A study of antifungal activity by a potential biological control strain, *Pseudomonas aureofaciens* strain PA147-2

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CONTENTS

FIGURES and TABLES

SUMMARY

In iron-rich conditions, *Pseudomonas aureofaciens* PA147-2 produces an antibiotic-like compound that inhibits the growth of a number of plant fungal pathogens. To contribute to the potential use of PA147-2 as a biocontrol organism, I report the identification of a genetic locus important for antibiotic biosynthesis. Mutants defective for fungal inhibition (Af^-), were generated by Tn5 mutagenesis. Southern hybridisation of genomic DNA from the Af^- mutants indicated that in each case loss of fungal inhibition was due to a single Tn5 insertion. Restriction mapping of cloned DNA showed that in two mutants, PA1 and PA109, the Tn5 insertions were in the same 16kb *Eco*RI fragment, separated by 2.1kb. Allele replacement, by homologous recombination with cosmids from a genomic library of PA147-2, restored one mutant (PA109) to antifungal activity. The 16kb *Eco*RI wildtype fragment complemented PA109 and PA1 *in trans* to antifungal activity. Saturation mutagenesis using a miniTn10 transposon identified a region of at least 13kb that is required for fungal inhibition in culture. Under growth room conditions, PA147-2 protected asparagus seedlings from *Phytophthora megasperma* root rot while the Af^- mutant, PA109, did not suppress the pathogen. An inhibitory compound isolated from PA147-2 and analysed by HPLC, was absent in three Af^- mutants. The region flanking Tn5 insertion in PA109 was partially sequenced. Thia region in mutant PA109 has significant homology to several two-component regulators and appears to be necessary for wildtype antifungal activity on PBPDA and *in planta*. The homologous wildtype region was sequenced and a single open reading frame encoding a putative regulatory protein was identified as a "hybrid kinase", a member of the family of two-component signal proteins in bacteria. The regulatory

gene is located within the putative cluster that is involved in antibiotic-mediated biological control of fungal pathogens.

CHAPTER 1

INTRODUCTION

The study of biological control of plant fungal pathogens in the soil environment by microorganisms is an area of growing scientific interest. An increasing awareness, and intolerance of the longterm effects of chemical residues in the soil, has resulted in the need for alternative agricultural practices that are less destructive on the environment. For biological control to have commercial success there needs to be a greater understanding of the disease control mechanisms by microbes and host plants at both the molecular and soil level. An ideal antagonistic microorganism must be able to compete and colonise aggressively in the rhizosphere, that is the soil surrounding the root system, in order to survive in a nutritionally limited environment. It must also successfully colonise the sites of pathogen infection such as wounds and lesions to prevent the establishment of the pathogen on the plant (Schroth and Hancock, 1982, Weller 1988, Chet, 1987).

Fluorescent pseudomonads are common rhizosphere inhabitants and contribute to the control of many deleterious fungal diseases (Fravel, 1988). Pseudomonads are ideal biocontrol antagonists because of their adaptive metabolism and ability to produce an array of inhibitory compounds. When introduced as seed or seedling inoculants, they can grow with the advancing root and successfully colonise and compete for nutrients, to the exclusion of the pathogen (Schroth and Hancock, 1982, Thomashow and Weller, 1990).

Many potential biocontrol microbes are found on the plant roots at the site of pathogen infection. They inhibit the growth of pathogens by producing secondary metabolites such as iron-scavenging compounds, antibiotics and

volatile substrates, which act in combination to inhibit pathogen establishment (Burr and Caesar, 1984, Davidson, 1988, Schippers 1988). This chapter will review the processes and implications of biological control of plant pathogens by fluorescent pseudomonads, from the recognition of disease suppressive soils to the elucidation of specific strains responsible for resistance, and current efforts to isolate the genetic determinants involved in biocontrol.

LITERATURE REVIEW

1.0 Antifungal activity by fluorescent *Pseudomonas* sp.

1.1 Disease suppressive soils

Biological control has been observed in agricultural systems since at least the turn of the century, and is defined as "the reduction in disease by one or more living organisms other than the host or man" (Baker, 1968). Despite early observations that biological control in some cases appeared to account for the absence of fungal diseases, it was a considerable length of time after its discovery that research in this area was stimulated in earnest. The reluctance to accept the application of biological control principles in agriculture was largely due to scepticism about whether it could provide a significant improvement on control achieved through traditional chemical and cropping practices (Baker, 1964, Baker, 1987, Bruehl, 1975).

It is now readily accepted that many soils can naturally suppress plant diseases caused by major fungal pathogens, including *Gaeumannomyces graminis* (Smiley 1979), *Pythium* (Hancock 1979), *Rhizoctonia* (Elad *et al.*, 1980), *Phytophthora* (Broadbent and Baker, 1974) and *Fusarium* spp. (Scher and Baker, 1980). Some soils remain naturally disease resistant or become so, after prolonged exposure to the pathogen (Baker and Chet, 1982). Crops have a tendency to be susceptible to disease when grown in newly cultivated areas. To control the onset of disease, practices such as rotating crops with less susceptible hosts, tillage and soil amendments with organic fertilisers are recommended to reduce disease severity. In some situations where susceptible land has been worked over long time periods, the soil gradually becomes resistant to disease. This is especially common

in conjunction with crop monoculture such as the case of wheat (Huber and Schneider, 1982, Shipton, 1975).

A soil is considered to be conducive when it supports a significant level of disease, whereas a suppressive soil is characterised by the non-development of soil-borne pathogens (Schroth and Hancock, 1982). Complex factors such as chemical and physical properties of the soil, as well as resident microflora, influence the nature and degree of suppressiveness (Schneider, 1982). Suppressive soil may be mixed into conducive soil and render the latter inhibitory to disease (Shipton *et al.,* 1973). The addition of 5% or more suppressive soil was required to induce resistance to tobacco root rot in a conducive soil (Stutz *et al.,* 1986). Similarly, when fine sandy loam soil that was suppressive to *Fusarium*-wilt was added to conducive clay loam soil, the incidence of wilt disease was significantly reduced (Scher and Baker, 1980). A decrease in the severity of take-all by *G. graminis* occurred when suppressive soil was transferred to sterilised soil in both greenhouse and field trials (Shipton, 1977). In each case, the suppressive effect was lost from soils that were heat-treated or fumigated, suggesting the ability to resist pathogens results from biological factors inherent in the soil. Suppressive soils that are induced by the inoculation of microorganisms may only be expressed after several crop generations and soil condition may determine whether or not the soil is naturally suppressive, a factor which is usually expressed from the outset (Schroth and Hancock, 1982). Within these situations of induced or natural suppression, a continuum exists from completely non-inhibitory to completely pathogen-suppressing (Baker, 1987, Cook and Baker, 1983).

1.2 Fluorescent pseudomonads in suppressive soils

Identifying the causal agents involved in soil suppressiveness has not always been definitive. While specific microbes can be identified as responsible, other factors, both biotic (competition, parasitism) and abiotic (soil pH, texture, geography, climate) influence the behaviour of the antagonist (Baker and Chet, 1982). The causal agent should be capable of multiplication to determine whether a suppressive soil results results from biological rather than physical factors (Baker and Chet, 1982). Therefore, if small amounts of suppressive soil induce resistance when added to a conducive soil, a biological entity is most likely involved. Fluorescent pseudomonads are the most commonly implicated bacteria mediating soil suppressiveness, although *Trichoderma harzianum* was found to be responsible for suppression of fungal disease caused by *Sclerotium rolfsii* and *Rhizoctonia solani* (Liu and Baker, 1980, Elad *et al.*, 1980).

Cook and Rovira (1976) investigated whether a general or pathogen-specific type antagonism accounted for *G. graminis* suppression in certain soils. They found :

i) higher numbers of pseudomonads in suppressive soils compared with conducive soils,

ii) heat treatment of the soil at 60°C for 30 min resulted in the loss of suppression; this treatment would eliminate pseudomonads but not sporulating *Bacillus* and many Actinomycetes,

iii) reinoculation of the soil with *Pseudomonas* isolates gave equal or better suppression compared to uninoculated soils. Similarly, highly antagonistic pseudomonads were recovered from take-all suppressive soils, but not from non-suppressive soils (Smiley, 1979).

Stutz *et al.*, (1986) examined the importance of naturally occurring rhizosphere bacteria and non-pathogenic strains of *Thielaviopsis basicola* in the control of tobacco black root rot. However, high numbers of fluorescent pseudomonads, and

not non-pathogenic *T. basicola*, were isolated from suppressive soils in different locations, suggesting that pseudomonads were responsible for soil suppressiveness rather than intra-specific fungal antagonism. Furthermore, disease suppression was negatively correlated with the absence of fluorescent pseudomonads from conducive soils and heat-treated suppressive soil. Pseudomonads isolated from *Fusarium*-wilt suppressive soil significantly reduced take-all in wheat (Wong and Baker, 1984). The inoculation of conducive soil with these fluorescent bacteria significantly decreased the incidence of wheat root rots. However, while many isolates showed antagonism of the fungal pathogens in culture, Wong and Baker (1984) showed there was no correlation between antibiosis on agar plates and disease suppression in the glasshouse, suggesting that *in vitro* antibiosis alone does not indicate a biological control potential *in planta*.

1.3 Soil factors and suppressiveness

The absence of disease may not necessarily indicate suppressive activity by antagonistic microorganisms. Soil type and composition, such as nutrient and chemical content, are extremely important in the establishment of suppressiveness (Lyda, 1982). Physical properties such as particle size, soil structure and porosity, root depth, and soil moisture retention influence the movement of both pathogen and antagonist (Broadbent and Baker, 1974, Scher and Baker, 1982). In one study, soil type appeared to affect establishment of *Fusarium*-wilt on flax, as disease formation was suppressed in clay-type soils but prevalent in sandy soils such as silt loams (Scher and Baker, 1980). Furthermore, while steam treatment or lowering the pH from 8.0 to 6.0 eliminated the suppressive effect, there was no significant difference in the severity of disease in conducive soil between pH 8.0, 7.0 and 6.0. In California sandy loam suppressiveness could be reduced by lowering the pH thereby increasing the availability of iron. This

resulted in about 20% of diseased flax seedlings at pH 8.0, about 60% at pH 7.0, and about 82% at pH 6.0 (Scher and Baker, 1980).

The importance of iron (Fe^{3+}) competition was demonstrated when addition of a synthetic Fe^{3+} chelator, ethylenediaminedi-*O*-hydroxyphenylacetic acid (EDDHA) induced suppression in a *Fusarium*-conducive soil (Scher and Baker, 1982). Disease suppression also occurred when *Pseudomonas putida* was added to conducive soil. Fe^{3+} is required for germ tube elongation in *Fusarium* spp., and the addition of *P. putida* and/or EDDHA limited the available Fe^{3+}, thereby reducing disease formation. Stutz *et al.,* (1986), however, acknowledged the presence of other factors, peculiar to the soils tested, that were somehow involved in disease suppression. A fluorescent pseudomonad, isolate CHAO, that induced suppression in most of the soils tested, had no effect in three conducive soils and could not be recovered from these soils. Furthermore, different levels of suppressiveness were observed in the conducive soils that did not support CHAO growth (Stutz *et al.,* 1986).

1.4 Biological control *in situ*

Since the discovery that resident soil microbes are involved in disease suppression, research has focused on identifying specific antagonists for disease control. For example, bacterial isolates, recovered from wheat roots growing in suppressive soil, were selected on the basis of *in vitro* inhibition of the pathogen (Weller and Cook, 1983). Coating wheat seeds with fluorescent pseudomonads resulted in protection against take-all in the greenhouse and in the field. *Pseudomonas fluorescens* 2-79 applied to wheat seeds, suppressed take-all in the glasshouse and the field (Weller and Cook, 1983). However, bacterial populations remained stable on the seeds at temperatures between 5-15°C, but rapidly decreased when the soil temperature was raised to 25°C. Native strains of *P. fluorescens* inhibited peanut root and stem rot caused by *Sclerotium rolfsii*

(Ganesan and Gnanamanickam, 1987). In glasshouse tests, 99% of the plants were protected from *S. rolfsii* infection if they were inoculated with *P. fluorescens*. This strain also inhibited the pathogen *in vitro*. Further examples where control of fungal disease has been achieved by specific fluorescent pseudomonads in the glasshouse and/or the field are given in Table 1.1.

A problem that is often encountered when soil isolates are screened for their potential as biocontrol agents is the lack of correlation between *in vitro* and *in vivo* assays for disease suppression. Weller and Cook (1983) found a positive correlation between *in vitro* inhibition and protection against take-all in the soil. However, it is generally considered that *in vitro* antagonism alone is not a sufficient basis for selection of potential biocontrol agents (Fravel, 1988). An initial search for putative antagonists should be carried out in soils where disease suppression can be observed (Weller *et al.*, 1985). The importance of this was illustrated by the lack of correlation between rhizosphere competence and root colonisation, and the production of antibiotics against *Rhodolorula glutinis* on potato dextrose agar (PDA) (Juhnke *et al.*, 1987). Furthermore, *Xanthomonas maltophilia,* which did not show antibiosis in culture, produced the greatest increase in yield of spring wheat compared to *Bacillus*, *Streptomyces* and a fluorescent *Pseudomonas* spp. in field trials.

The numerous examples of successful control of many pathogens by specific pseudomonads is an indication of their importance as potential biological control agents (Table 1.1). Baker and Chet (1982) suggest that in many field trials, an important control is often overlooked. This involves the inoculation of plant material with a bacterial strain, identical in every way to the antagonist except lacking a property that is suspected to be responsible for antagonism *in situ*. This requirement is fulfilled by including mutants in glasshouse and field assays, that are identical to the wild-type but deficient in a putative biocontrol mechanism.

This approach depends upon reliable laboratory assays to screen for putative mutants, such as for siderophore and antibiotic synthesis *in vitro*. However, it is more difficult to screen for mutants defective in root colonisation and competition *in vitro* as approximating complex soil conditions and microbial interactions becomes extremely difficult.

Table 1.1

Reports of the application of specific fluorescent pseudomonads to control fungal disease

Pathogen	Antagonist	Disease control	Source
Rhizoctonia	*P. cepacia*	Wheat	de Freitas, 1991[1]
	P. putida " "		
	P. luorescens	Cotton	Howell, 1979[2]
Fusarium	*P. cepacia*	Onion	Kawamoto, 1976[3]
	P. putida	Flax, cucumber radish	Scher, 1982[4]
Pythium	*P. fluorescens*	Pea	Parke *et al.*, 1991
	" "	Wheat	Weller, 1986[5]
	" "	Cotton	Howell, 1980[6]
Aphanomyces	" "	Pea	Parke *et al*, 1991
T.basciola	" "	Tobacco	Laville *et al*, 1992
G.graminis	" "	Wheat	Brisbane, 1988[7] Weller, 1983
	P. fluorescens-putida group	" "	Wong, 1984[8]
Sclerotium rolfsii	*P. fluorescens*	Peanut	Ganesan, 1991[9]
Sclerotinia sclerotium	*P. cepacia*	Sunflower	Mcloughlin, 1992

Key to references

[1]de Freitas and Germida, 1991. [2]Howell and Stipanovic, 1979. [3]Kawamoto and Lorbeer, 1976. [4]Cher and Baker, 1982. [5]Weller and Cook, 1986. [6]Howell and Stipanovic, 1980. [7]Brisbane and Rovira, 1988. [8]Wong and Baker, 1984. [9]Ganesan and Gnanamanickam, 1991.

1.5 Mechanisms of biological control

Fluorescent pseudomonads employ a number of specific mechanisms to suppress pathogens. In general these mechanisms include production of siderophores, antibiotics, volatile substances such as hydrogen cyanide (HCN), competition for essential nutrients, and colonisation and induction of systemic resistance in plants through the application of plant growth-promoting rhizobacteria (PGPR). Some or all of these mechanisms may be involved in the process of antagonism (O'Sullivan and O'Gara, 1992).

1.6 Siderophores

Fluorescent pseudomonads are characterised by their ability to produce yellow-green fluorescent compounds under conditions of iron limitation (Stanier *et al.*, 1966). These compounds, termed siderophores, fluoresce under short wave UV, selectively complex with Fe^{3+} with little or negligible affinity for Fe^{2+}, and are involved specifically in iron uptake into the cell (Leong, 1986). One class of siderophores, known as pyoverdines or pyoverdins, have been characterised using nuclear magnetic resonance (NMR) and mass spectrophotometry (Leong, 1986). The compounds within this group are all chromopeptides, that is a peptide chain of 6-8 amino acids bound to a chromopohore derived from 2,3-diamino-6,7-dihydroxyquinoline (Teintze *et al.*, 1981). Meyer and Hornsperger (1978) demonstrated a specific role for pyoverdin in Fe^{3+} binding and transport. The addition of purified pyoverdin from *P. fluorescens* to the external medium resulted in a significant increase in the rate of iron uptake by the cells. The exact structure of one pyoverdin, known as pseudobactin, has been determined for *P. fluorescens* strain B10 (Kloepper *et al.*, 1980a, Teintze *et al.*, 1981). The fluorescent pseudomonads are thought to produce related pseudobactin-like siderophores that differ principally in the number and configuration of amino acids in the peptide chain (Neilands and Leong, 1986).

While siderophore production may be a ubiquitous property of the fluorescent pseudomonads, the compounds themselves are chemically and structurally diverse among other microorganisms. Fungi, for example, generally produce siderophores of the hydroxamate Fe^{3+}-binding groups while enteric bacteria produce siderophores with catecholate iron (III)-binding ligands (Neilands and Leong, 1986). Other phytopathogens may not have the iron assimilation systems and receptors of the PGPR (Schippers *et al.,* 1987). Therefore, a siderophore from one *Pseudomonas* strain may not be recognised by another. Buyer and Leong (1986) demonstrated that bean-specific strains of deleterious *Pseudomonas* sp. were inhibited by beneficial *Pseudomonas* strains. This was in part due to the inability of the deleterious strains to utilise the siderophores produced by the beneficial pseudomonads. This results from the presence of outer membrane receptor proteins, that specially recognise the producer's own ferric-siderophore. A structural gene (localised to a 2.4kb *Eco*RI fragment) that encoded an 85 kDa outer membrane receptor protein for ferric pseudobactin was isolated from a genomic library (Magazin *et al.,* 1986). The introduction of a cosmid containing the structural gene into a bean-deleterious and bean-beneficial *Pseudomonas* strain, both of which are normally inhibited by *P. fluorescens* B10 and pseudobactin, enabled these strains to uptake ferric pseudobactin and therefore utilise the iron-siderophore for growth.

1.7 Siderophores and biological control

Siderophores function in biological control by sequestering iron in the root zone, making it unavailable to certain rhizoplane microorganisms and inhibiting their growth. Kloepper *et al.,* (1980c) demonstrated that *Fusarium*-wilt or take-all conducive soil could become suppressive by the addition of either *P. fluorescens* B10, or its purified siderophore pseudobactin. Furthermore, addition of iron to a soil normally suited to *Fusarium* suppression, caused the soil to become conducive. This 0suggests that siderophores and iron competition are involved in *P.*

fluorescens B10-mediated disease control. Similarly, when a *P. putida* strain, or iron-chelating agents that mimic the action of siderophores, were added to *Fusarium*-conducive soil, the soil became suppressive to flax, cucumber and radish wilt pathogens (Scher and Baker, 1982).

Pseudomonas strain B324 promoted the growth of wheat *in vivo* and inhibited seven different isolates of *Pythium* on Kings B Medium (KBM), a low iron medium suitable for siderophore production. In contrast, this strain showed no inhibition of the pathogens on iron-rich medium (Becker and Cook, 1988). The inhibition on KBM could also be nullified by addition of $FeCl_3$ suggesting that antagonism *in vitro* is due to the action of siderophores rather than antibiotics. Fungal inhibition *in vitro* positively correlated with *in vivo* results: five siderophore-negative mutants showed no significant growth response or *Pythium* suppression on wheat compared to the positive growth promotion observed with the wild-type. Furthermore, addition of an iron chelator (EDDHA) resulted in growth promotion equal to that obtained with the wild-type, strongly supporting a role for siderophores in *Pythium* suppression and growth promotion. The ability for the purified siderophore to promote growth and protect wheat against *Pythium* could also be investigated in future studies.

Transposon mutants were used to determine a role for siderophore production by a *P. fluorescens* strain which inhibits *Pythium* damping-off of cotton. The mutants selected were either unable to fluoresce on KBM (Flu^-) or unable to grow on KBM supplemented with EDDHA (Sid^-) (Loper, 1988). The wild-type strain decreased *Pythium* colonisation and increased cotton seedling emergence; however the mutants did not, suggesting siderophores may account for some of the observed biological control.

However, some caution should be taken when interpreting results from experiments with siderophore-negative mutants. A mutation affecting an important nutritional factor, such as iron, may have masked effects on cellular processes other than siderophore production. For example, some fluorescent pseudomonads produce HCN which inhibits cellular metabolism (Bakker and Schippers, 1987). Approximately 50% of the potato rhizosphere bacteria isolated produced HCN *in vitro*. Furthermore, HCN production in one deleterious rhizosphere microorganism (DRMO) was dependent on Fe^{3+} concentration. Some growth promoting pseudomonads, including a strain WCS358, did not produce HCN, which suggests siderophore-producing bacteria may compete and inhibit DRMO by limiting the iron availability for HCN production. The maintenance and competition for a single nutrient such as iron probably occurs on many different levels in the soil rhizosphere and involves complex interactions between microorganisms.

1.8 Plant Growth-Promoting Rhizobacteria (PGPR)

The term PGPR refers to those soil bacteria that indirectly enhance plant growth by inhibiting pathogens through competition for limited nutrients and induction of the hosts natural defences (Schippers, 1988). PGPR strains may also produce hormones or metabolites that directly influence plant growth. Loper and Schroth (1986) examined the effect of bacterially-produced indole acetic acid (IAA) on the roots of sugarbeet seedlings. Bacteria producing the highest levels of IAA in culture also increased root:shoot ratios and decreased root elongation on sugarbeet seedlings. Inoculation of seeds with an IAA producing *P. syringae* isolate resulted in a decrease in root elongation while a non IAA-producing mutant had no effect on roots. PGPR strains may also work in combination with other soil microbes to induce a growth response. A *P. putida* strain, in combination with naturally occurring vesicular-arbuscular mycorrhizal (VAM) fungi, significantly increased plant growth and nodulation in clover (Meyer and Lindeman, 1986).

Plant shoots also showed greater concentrations of Fe, Cu, Al, Zn, Co and Ni ions. There are considerable benefits to those plants able to form associations in this manner. While VAM fungi on their own did not significantly influence plant growth, the enhanced VAM uptake of minerals, perhaps solubilised by PGPRs could account for the observed growth promotion and increased nodule formation (Meyer and Lindeman, 1986). Furthermore, these types of microbial associations should not be overlooked when considering the mechanism of growth promoting by various pseudomonads.

Siderophores play an important indirect role in plant growth stimulation by suppressing disease causing pathogens (Elad *et al.,* 1987, Weller and Cook, 1986). Kloepper *et al.,* (1980b) demonstrated a growth promotion effect by *P. fluorescens* B10 on field tests of potato, radish and sugarbeet. An increase in plant growth of up to 144% was due to pseudobactin-mediated inhibition of *Erwinia carotovora.* However, there was no growth promotion when PGPR-inoculated potatoes were planted in soil amended with FeEDTA. Significant growth promotion occurred on apple seedlings and rootstocks treated with PGPR both in the glasshouse and the field (Caesar and Burr, 1987).

Siderophore production may contribute to but not always account for observed biological control. *P. fluorescens* 2-79 produces pyoverdin and the antibiotic phenazine-1-carboxylate (PCA), and an earlier study showed phenazine accounted for some, but not all, of the biological control observed in the wheat rhizosphere (Thomashow and Weller, 1988, 1990b). In a recent study to define a role for pyoverdin production by 2-79, siderophore-negative mutants were constructed and tested for take-all suppression (Hamdan *et al.,* 1991). These mutants controlled take-all as effectively as parental wild-type strains both *in vitro* and in soil, suggesting that PCA was the dominant factor for disease control, and that siderophores have little or no role at all. *P. fluorescens* 2-79 also appears to

produce another antifungal factor (Aff), usually masked by siderophore production, that may be responsible for the fungal inhibition previously ascribed to siderophores in PCA-negative mutants (Weller *et al.,* 1986). The antifungal factor, possibly anthranilate, was identified as an orange halo present on low iron media (Hamdan *et al.,* 1991). Some mutants deficient for PCA, siderophores and Aff were still able to cause a small reduction in take-all, indicating the involvement of additional mechanisms for fungal inhibition (Hamdan *et al.,* 1991).

Paulitz and Loper (1991) could not attribute a significant role to siderophores from *P. putida* for the control of *Pythium* in three agriculturally different soils. Pyoverdin-negative mutants were unable to inhibit mycelial growth on KBM, however, they were not significantly different from the wild-type in protecting cucumber seedlings from *Pythium* disease. This study contradicts an earlier finding (Loper, 1988) that suggested a significant role for siderophores in *Pythium* control. However there were a number of experimental differences, including the strain of *Pseudomonas* used (*P. putida* and *P. fluorescens*), the pathogenic *Pythium* isolates, soil type, temperature (23°C and 20°C), host plant (cucumber and cotton) and emergence durations from planting (Paulitz *et al.,* 1991, Loper, 1988). These and other factors contribute to the complexity of the task of assigning specific roles to the various factors mediating biocontrol.

Siderophore compounds may have a more direct effect on a pathogen, rather than indirectly starving them of iron. Siderophores produced by *P. fluorescens* CHAO were more toxic when complexed with Fe^{3+} and strongly inhibited mycelial growth of *T. basicola* (Ahl *et al.,* 1986). This may be due to the Fe(III)-complexed siderophores increasing the intracellular iron to toxic levels in *T. basicola* as iron-free siderophores did not inhibit mycelial growth or spore germination. Strain CHA0 also produces antibiotics and HCN, but how these mechanisms function in the soil and which of these is more important has yet to be

determined. However, it is possible that all three mechanisms, and perhaps others, act in combination *in situ.*

1.9 Pseudobactin B10 and Pseudobactin 358

The genetic organisation of siderophore production has been comprehensively studied for both pseudobactin 358 (PB358) and pseudobactin B10 (Marugg *et al.,* 1988, Moores *et al.,* 1984). *Pseudomonas putida* WCS358 promotes growth and depresses the effect of narrow crop rotation associated with potato, by production of PB358 (Geels and Schippers, 1983). Tn5 mutagenesis and complementation with genomic library cosmids has defined at least five separate gene clusters involved in siderophore biosynthesis and transport (Marugg *et al.,* 1985). Two Flu^- Sid^- mutants, unable to produce PB358 *in vitro,* were also unable to promote growth of root stem cuttings in short potato rotation soil compared to the wild-type (Bakker *et al.,* 1987), which supports a role for PB358 in growth promotion. A major gene cluster for pseudobactin 358 (gene cluster A) covers 33.5 kb of DNA and is complemented by six cosmid clones (Marugg *et al.,* 1988). This region alone contains at least five transcriptional units and some of these promoters are regulated by iron at the transcriptional level. The biosynthetic genes appear to be arranged in clusters throughout the genome.

In a similar study using mutants deficient in pseudobactin B10 production, a cosmid complementation pattern suggests at least twelve genes organised in four clusters are required for biosynthesis of this siderophore (Moores *et al.,* 1984). An 85 KDa receptor protein gene lies in the midst of a locus containing pseudobactin B10 biosynthetic genes (Magazin *et al.,* 1986). Similarly, a minimum of four genes arranged in clusters throughout the genome are involved in the production of a fluorescent siderophore from *P. syringae* (Loper *et al.,* 1984). The presumed biochemical complexity of siderophore systems appears to be consistent with

current findings of the genetic complexity involved in the production of both B10 and PB358 (Gutterson, 1990).

1.10 Antibiotics

Pseudomonads produce an array of secondary metabolites and compounds, many of which have antibiotic, and more specifically, antifungal activities (Lesinger and Margraff 1979, Lievens *et al.,* 1989). However, the production of antibiotics in the soil and their role in disease control has been questioned particularly with respect to whether they are produced in sufficient quantities to be effective against fungi (Gottlieb, 1976). Furthermore, antibiotics are readily inactivated by biodegradation and adsorption onto clay particles and organic matter (Gottlieb, 1976, Williams and Vickers, 1986).

1.11 Antibiotics in biocontrol

Many pseudomonads produce antibiotics in culture and in some cases this has positively correlated with biocontrol in the glasshouse and field. Furthermore, the purified antibiotic or cell free culture filtrates often mimics the biocontrol activity of the whole agent. For example, Howell and Sipanovic (1979) isolated the antibiotic pyrrolnitrin from *P. fluorescens* and demonstrated *in vitro* antibiosis against *R. solani, T. basicola, Alternaria* sp, and *V. dahliae.* Cotton seedling survival was increased from 30 to 70% when seeds were treated with either *P. fluorescens* or pyrrolnitrin and planted in *R. solani* infested soil. The same authors also isolated pyoluteorin from another *P. fluorescens* strain. Both the strain and this antibiotic had no effect on *R. solani* but inhibited *Pythium ultimum* and reduced seedling disease (Howell and Stipanovic, 1980). Pyrrolnitrin was shown to be important for *P. fluorescens* BL915 control of *R. solani*-induced damping-off of cotton (Hill *et al.,* 1994). A genomic region restored pyrrolnitrin production to a number of mutants and conferred antibiosis ability on two other *P. fluorescens* strains, not otherwise known to produce this compound (Hill *et al.,*

1994). Recent results suggest this genomic region contains a global regulator of a number of antifungal metabolites (Lam *et al.*, 1994). A number of antibiotics, including pyrrolnitrin, were isolated from *P. cepacia*, and shown to inhibit diseases such as radish damping-off, tomato *Fusarium*-wilt and eggplant *Verticillium*-wilt (Homma *et al.*, 1989). Several novel peptide antibiotics, collectively called Pseudomycins, were isolated from a *P. cepacia* strain and were shown to inhibit a broad range of fungal pathogens *in vitro* (Harrison *et al.*, 1991).

1.12 Genetic evidence for the role of antibiotics in biocontrol

Based on the evidence described in the preceding section, and other studies, it is now accepted that antibiotic production by pseudomonads has a significant role in biological control (Weller *et al.*, 1990, Gutterson, 1990). This conclusion is strongly supported by genetic studies with antibiotic-deficient mutants as appropriate controls. Significant control of Dutch Elm disease which is caused by *Ceratocystis ulmi*, was achieved with dual inoculation of elm tree seedlings with the pathogen and an antimycotic-producing antagonist, *P. syringae* (Lam *et al.*, 1987). However, a Tn903-generated mutant deficient in antibiotic production did not control the onset of disease. All introduced strains could also be reisolated after two growing seasons, and were shown by Southern hybridisation to contain the transposon at the same locus.

An antibiotic, 2,4-diacetylphloroglucinol (DAPG), from a *Pseudomonas* isolate inhibits *Rhizoctonia*, *Pythium* and *Fusarium* pathogens in both high and low-iron media (Shanahan *et al.*, 1992). A mutant of this strain, for which HPLC failed to detect the antibiotic, also failed to inhibit these fungi *in vitro*. The production of 2,4-diacetylphloroglucinol is also an important mechanism for inhibition of tobacco root rot by *P. fluorescens* (Keel *et al.*, 1990b) and take-all of wheat by *P. aureofaciens* (Vincent *et al.*, 1991). In both cases a positive correlation between

antibiotic production and disease control is supported by the isolation of mutants that are restored to wild-type activity by cosmid complementation.

Two strains of *P. cepacia* increased sunflower emergence in the presence of the wilt-causing pathogen, *Sclerotium rolfsii,* in the glasshouse (McLoughlin *et al.,* 1992). One *P. cepacia* strain produced three biologically active compounds *in vitro,* pyrrolnitrin, aminopyrrolnitrin and monochloroaminopyrrolnitrin. However, four antibiotic-negative mutants were not significantly different from the wild-type in disease suppression *in vivo*, suggesting that in this case, antibiotic production is not significant in the biocontrol of sunflower wilt. Treatment of cotton seeds with pyoluteorin or pyrrolnitrin produced by Pf-5, protected cotton seedlings against *P. ultimum* (Howell and Stipanovic, 1980) and *R. solani* (Howell and Stipanovic, 1979), respectively. However, pyoverdin (Pyd^-) and pyoluteorin (Plt^-) mutants were similar to the wild-type in suppression of *Pythium* control on cucumber (Kraus and Loper, 1992), suggesting these two metabolites do not have a significant role. Furthermore, pyoluteorin-overproducing mutants (Plt^{++}), and Tn5-mutants unable to produce three antifungal compounds including pyrrolnitrin, were also similar to the wild-type in suppression of *Pythium* damping-off. Mechanisms such as physical exclusion and nutrient competition may be more predominant in Pf-5-*Pythium* control than the production of antifungal metabolites.

1.13 Phenazine-1-carboxylate

Control of the take-all pathogen of wheat, *G. graminis* var. *tritici* (Ggt), has focused more recently on suppression of the disease using fluorescent pseudomonads (Weller and Cook 1983, Wong and Baker *et al.,* 1984, Wilkinson *et al.,* 1985, Poplawsky *et al.,* 1988, Poplawsky and Ellingboe 1989, Ryder *et al.,* 1990, Thomashow and Weller 1990a, 1990b, Thomashow and Pierson, 1991). *P. fluorescens* strain 2-79 produces the antibiotic phenazine-1-carboxylate (PCA)

(Brisbane *et al.*, 1987) that is important for suppression of take-all on roots (Thomashow and Weller, 1988). *LacZ* fusion constructs were used to examine the regulation of phenazine synthesis in a related *P. fluorescens* strain 30-34 (Thomashow *et al.*, 1991). Phenazine production was inhibited by glucose and ammonia, but was stimulated with nitrate as the sole source of nitrogen. Iron also induced gene-fusion expression by five to eight fold.

1.14 Oomycin

P. fluorescens HV37a inhibits *P. ultimum* on both cotton seedlings and *in vitro* (Gutterson *et al.*, 1986, Gutterson, 1990b). HV37a mutants deficient for fungal inhibition were complemented by library cosmids in a pattern that indicates the involvement of at least five genes involved in antibiotic biosynthesis (Gutterson *et al.*, 1986). HV37a produces an antibiotic, oomycin, on PDA and an additional two antibiotics on potato agar (PA) in the absence of glucose (James and Gutterson, 1986). Oomycin A accounts for approximately 70% of the ability of HV37a to reduce *Pythium* root infection of cotton, and 50% of its ability to increase cotton seed emergence (Howie and Suslow, 1991). Mutations in the *afuA* and *afuB* loci of HV37a are deficient in glucose dehydrogenase and oomycin A production. The *afuE* gene which is required for oomycin A synthesis is regulated by glucose at the transcriptional level, although an additional factor is also required for regulating antibiotic production (Gutterson *et al.*, 1988). Oomycin A is not expressed fully in the cotton spermosphere, the soil environment surrounding the seed, until 12h after seed imbibition (Gutterson, 1990a). The antibiotic is produced more rapidly on seed surfaces by derivatives of HV37a that produce oomycin A constitutively, rather than by the wild-type strain. The constitutive producers are more effective than HV37a at suppressing *Pythium* damping-off on cotton, probably because the

rapid accumulation of antibiotic reduces *Pythium* colonisation on the cotton seeds immediately after imbibition (Gutterson, 1990a).

1.15 2,4-Diacetylphoroglucinol (DAPG)

A number of studies have examined the significance of DAPG production in fungal inhibition (Gutterson, 1990a). A 22-kb DNA fragment from *P. fluorescens* CHAO complements DAPG-minus mutants (Haas *et al.*, 1991), however, the organisation and regulation of these genes is still to be reported. *P. fluorescens* strain CHAO produces several secondary metabolites involved in suppression of fungal disease, such as HCN (Voisard *et al.*, 1989), pyoluteorin (Ptl) (Maurhofer *et al.*, 1992), and 2,4-diacetylphloroglucinol (Phl) (Keel *et al.*, 1992). Mutants defective in the synthesis of Phl no longer controlled black root rot of tobacco (Defago and Haas 1990, Keel *et al.*, 1990b) or take-all of wheat (Keel *et al.*, 1992). A 22-kb fragment isolated from genomic library of the wild-type CHAO, enhanced the production of Ptl and Phl but not HCN *in vitro* (Maurhofer *et al.*, 1992). This increased antibiotic activity accounted for protection of cucumber plants from *P. ultimum.* However overproduction of these antibiotics had toxic effects on sweetcorn and cress (Maurhofer *et al.*, 1992). Production of DAPG was also significant for the biological control of wheat (Shanahan *et al.*, 1992, Vincent *et al.*, 1991).

1.16 Antibiotic persistence in soil

Antibiotics have been detected in soil and on roots treated with various biological control agents, providing further evidence of their importance in disease suppression. Pyrrolnitrin was detected in the soil at least 30 days after bacterial inoculation, whereas pyoluteorin was quickly absorbed and inactivated by soil particles (Howell *et al.*, 1980). Wright (1956a, 1956b) isolated gliotoxin from soil sown with wheatstraw fragments that were inoculated with *T. basicola*, and suggested that antibiotics are produced in soil at microsites where there is an available supply of rich nutrients. Phenazine-1-carboxylate was detected in the

rhizosphere of wheat roots colonised by 2-79, whereas this antibiotic was not detected in the rhizosphere of wheat roots colonised with phenazine-negative mutants (Thomashow *et al.,* 1990b). This provides direct evidence for the production of an antibiotic on plant roots and its importance in the control of a particular fungal disease. Antibiotics are more easily detected at microsites, where nutrient sources such as root exudates and organic matter provide conditions which may favour the production of high local concentrations of antibiotic (Weller and Thomashow, 1990).

1.17 Root Colonisation and Competition

The ability to colonise and maintain population levels, as well as to successfully compete with other soil microbes, is an important attribute for PGPR bacteria. *Pseudomonas* sp. are aggressive rhizosphere colonisers and are physiologically well adapted to utilise a range of metabolic substrates for growth (Weller, 1988). There is continual competition for nutrients, particularly carbohydrates, oxygen, space and possibly growth factors, occurring between soil microbes (Baker and Cook, 1974). The ability to competitively exclude other microbes relates directly to a strains ability to colonise the root successfully. Essentially, all the disease-suppressive mechanisms exhibited by fluorescent pseudomonads are of no value unless the strain can establish and maintain itself on the roots. Assays to select for good competitors and colonisers cannot be easily carried out using *in vitro* screens, because agar tests do not accurately reproduce soil conditions or the competitive interactions (Lam, 1990). The most accurate evaluations of colonising ability of a particular strain are those conducted *in vivo.*

A positive correlation has been shown between plant growth promotion by *P. fluorescens* of potatoes, antibiosis, and a decrease of microflora populations in the root zone, suggesting that the increase in plant growth was due in part to

displacement of other root microorganisms (Kloepper *et al.,* 1980b, Kloepper and Schroth, 1981). Therefore, some growth-promoting pseudomonads may modify the composition of root microflora and thereby indirectly limit the disease effects of fungal pathogens. *P. fluorescens* E6 reduced the frequency of *Penicillium* root colonisation, but had no effect on total bacterial and fungal levels (Yeun and Schroth, 1986).

Mutants deficient in particular traits can be used to identify genetic factors involved in root colonisation and competition. Lam (1990) reported the isolation of *lacZ*-fusion mutants deficient in "competitive colonisation", i.e., mutants unable to successfully colonise roots in the presence of other microbes. Five mutants that were deficient for motility, were examined for their ability to colonise wheat seedlings in the presence of the wild-type parent. This resulted in wild-type:mutant ratios of 20:1 or greater, whereas the mutants were able to colonise wheat roots at levels similar to the wild-type inoculated alone (Lam, 1990).

The production of adhesion substrates such as agglutinin appear to be important for colonisation of a root system (Anderson *et al.,* 1988). Mutants defective in agglutinin production showed reduced colonisation ability, which also correlated with a reduction in protection of cucumber roots against *Fusarium*-wilt (Tari and Anderson, 1988). To date, there is only limited information on the complex factors involved in competition and colonisation. However, characterisation of these genes will provide important information for the development of improved strains for biological control.

1.18 Motility and Chemotaxis

To study the role of motility during colonisation, a selection of biocontrol agents were measured for their ability to move from inoculated seeds onto growing roots (Chao *et al.,* 1986). However none of the agents tested, including two fluorescent pseudomonads, were able to colonise the rhizosphere more than 2cm from the treated seed, although water aided vertical distribution to some extent (Chao *et al.,*

1986). The antagonists did not move onto the roots, however, a *P. putida* strain tested in this study had previously been shown to successfully colonise potato roots and increase plant growth (Kloepper *et al.,* 1980b). The lack of root colonisation ability associated with biocontrol (Chao *et al.,* 1986), is in contrast with another study reporting that *P. fluorescens* 2-79 successfully colonised wheat roots and maintained relatively stable populations throughout the growing season (Weller, 1983, 1984). Strain 2-79 was distributed along the entire length of the roots with higher populations nearer the root base.

Non-motile mutants of an antagonistic strain of *P. putida* were used to investigate the contribution of motility and chemotaxis in fungal inhibition on plants (Scher *et al.,* 1988). A non-motile mutant of *P. putida* was unaffected in its ability to colonise roots and showed the same distribution pattern along the roots as the motile parent (Scher *et al.,* 1988). Flagella also appeared to be unimportant for wheat root colonisation by *P. fluorescens* 2-79, since three non-motile mutants colonised roots as well as the motile parent (Howie *et al.,* 1987). These studies suggest that motility and chemotaxis are probably not required for root colonisation by these fluorescent pseudomonads. In contrast however, four flagella-minus mutants of the growth-promoting pseudomonad WCS374, were significantly impaired in their ability to colonise growing potato roots compared to the motile parent (DeWeger *et al.,* 1987).

A lack of consistency among these studies in defining the specific mechanisms involved in colonisation and competition is probably a reflection of complex variables such as different assay systems (e.g., *Pseudomonas* sp. and host plant), soil and growth conditions (e.g., nutrient amendments, space, soil porosity, greenhouse vs field). Such variables make it difficult to define the specific determinants for motility and chemotaxis in a single statement. However, these studies clearly illustrate the diversity of factors involved in the establishment of the antagonist at sites of pathogen infection.

1.19 Induced systemic resistance

PGPRs have been recognised for their positive effects on plant growth through direct growth promotion and/or the inhibitory effects of their secondary metabolites on phytopathogens. Systemically acquired resistance (SAR) can be induced by prior inoculation with pathogens (Tuzun and Kuc, 1985, Tuzun and Kloepper, 1994) or non-pathogens (Roveratti *et al.*, 1989), and seed treatment with specific PGPR strains (van Peer *et al.*, 1991). Wei *et al.*, (1991) tested 94 PGPR strains for their ability to control anthracnose, a cucumber foliar disease caused by *Colletotrichum orbiculare*. Six PGPR strains significantly reduced lesion diameter when the pathogen was applied 21 days after planting. Spatial separation of the pathogen and PGPR strain, and failure to reisolate inducing strains from the plants, suggests PGPR strains can act as inducers of SAR (Wei *et al.*, 1992). Furthermore, inoculation with beneficial microbes such as PGPRs has an obvious advantage over using pathogens as inducers (Tuzun and Kloepper, 1994).

1.20 Summary

Initial discovery of suppressive soils and resident antagonists led to the isolation of specific microbes, most commonly pseudomonads, that were able to control disease. In most instances the actual mechanisms of biocontrol have not be specifically defined. The use of molecular genetics, beginning with the isolation of mutants defective for biocontrol, provides a definitive approach to assign importance to a specific mechanism (O'Sullivan and O'Gara, 1992).

The definition of a pathogen suppressive soil was proposed by Baker and Cook (1974) as soils in which "pathogens cannot establish, they establish but fail to reproduce disease, or they establish and cause disease at first but diminish with culture of the crop". Bruehl (1987) suggests this definition is no longer valid, as it inappropriately includes those soils exhibiting general suppression, a

phenomenon that is the result of multiple factors. Instead, the term suppression should apply to situations where disease suppression is effective against only a single pathogen, and does not affect the severity of other diseases of the same host. As complex soil factors are not reproducible (Baker and Chet, 1982), only situations associated with microbial interactions are truly suppressive, and not physical and chemical factors. In the light of this, it is perhaps inappropriate to include examples such as soils not conducive to *Fusarium* wilt, where in some areas disease suppression is strongly dependent on soil type and pH (see 1.1.4 of this chapter). However, inclusion of these examples illustrates the complexity of a phenomenon such as soil suppressiveness and biological control. Although certain microbes may be present, they do not always account for the absence of disease.

There are many considerations for the application of biological agents to become common place in agriculture. Desirable traits for a biocontrol agent would include cost effectiveness, the efficiency of achievable control, the ability to maintain stable populations, and the ability to reproduce itself in successive seasons to decrease time and reinoculation costs. In most experimental situations where soil suppressiveness was induced through inoculation of fluorescent pseudomonads, the seeds were coated or roots treated with high inoculum concentrations of bacteria. For prolonged suppression, the inoculum may have to be repeatedly applied and organic soil amendments may be necessary. These conditions may make the application of large inocula of bacteria to crops non-viable.

Clearly there are many factors implicated in biological control that remain to be identified. The increasing number of inconsistent reports is further evidence of numerous, intricate and complex processes. Genetic studies have identified some of the genes for biosynthesis and organisation of siderophores and antibiotics. However, our molecular understanding of root colonisation factors is not as

advanced, as it is for siderophores and antibiotics. The increasing availability of improved genetic vectors for pseudomonads, which are often very recalcitrant to genetic manipulation will be of great assistance in this area.

Project aims

This study characterises a previously unidentified fluorescent pseudomonad, with respect to its ability to inhibit certain fungal phytopathogens. The aim of this project is to analyse antifungal activity by a fluorescent pseudomonad at a molecular level. A bacterial soil isolate identified as *Pseudomonas aureofaciens* 147-2, was chosen to investigate the genes involved in production of an antifungal antibiotic. This involved the following strategy:

1. ISOLATION OF ANTIBIOTIC-NEGATIVE TN*5* MUTANTS (Af^-)

2. GENETIC ANALYSIS OF Af^- MUTANTS

i) Cloning the Tn5 insertions from some mutants and selection of two Af^- mutants for further analysis

ii) Recovery of wild-type sequences from a genomic library and complementation and subcloning of a DNA region involved in antifungal activity

iii) Saturation-mutagenesis and genetic complementation of an antifungal region

iv) DNA sequence analysis of genetic loci required for fungal inhibition

3. BIOCHEMICAL CHARACTERISATION

i) Isolation and analysis of an antifungal compound from PA147-2 by HPLC

ii) Demonstration that the presence of Tn5 insertion results in loss of antibiotic compound from the mutants

5. ASSAY FOR BIOLOGICAL CONTROL

i) Determine the biological control ability of PA147-2 against a fungal pathogen in glasshouse assays

ii) to establish whether a correlation exists between *in vitro* and *in planta* fungal inhibition and to assign a role for antibiotic production by PA147-2 in disease suppression.

CHAPTER 2

Characterisation and mutagenesis of an antifungal pseudomonad

Introduction

There is growing evidence that antibiotics produced by many biological control strains inhibit phytopathogens. However, the role of these antibiotics has been difficult to define, since many of these compounds cannot easily be detected in the soil (Davidson, 1988). Through the application of recombinant DNA techniques and strategies for *in situ* detection, it is possible to assign a role to certain metabolites produced by biological control strains. For antibiotics, this has been achieved by identifying antibiosis-negative mutants, which are unable to inhibit fungal pathogens in laboratory culture and glasshouse and field trials, and establishing a correlation between antibiosis and disease suppression (Weller and Thomashow, 1990, Jayaswal *et al.,* 1992).

Isolation of antibiotic-deficient mutants of *P. fluorescens* has led to characterisation of the genes involved in biosynthesis of phenazine-1-carboxylic acid (Thomashow and Weller, 1988) and oomycin A (Gutterson, 1990). Recently, two antibiotics pyrrolnitrin and 2-hexyl-5-propyl-resorcinol were detected in barley seeds after application of biological control strains of *Pseudomonas* (Kempf *et al.,* 1994). Pyrrolnitrin was also found with adhering rhizosphere soil in cotton roots. However the higher antibiotic levels found in the barley spermosphere (the soil environment surrounding the seed) suggests that barley seed provides a richer nutrient supply for the biocontrol strain (Kempf *et al.,* 1994). *In situ* detection of antibiotics produced by biocontrol strains provides direct evidence of antibiotic production in the soil under natural conditions (Kempf *et al.,* 1993, Thomashow *et al.,* 1990b).

Disease caused by *Aphanomyces euteiches*

Soil bacteria, especially pseudomonads, may be useful as biocontrol agents against fungal pathogens which affect agricultural crops. *Aphanomyces euteiches* Drechs. is known worldwide, primarily for its destructiveness on pea (Jones and Dreschsler, 1925) and bean (Pfender and Hagedorn, 1982). The fungus invades the roots causing root damage, and in severe infection may cause wilting and necrosis to the leaves and stem of the plant. However, *Aphanomyces* root infection is not usually obvious above ground (Papavizas and Ayers, 1974). *A. euteiches* causes "damping off" and substantial damage to leguminous crops and more recently has been identified as a lucerne pathogen in Canada (Richard *et al.*, 1991, Beghdadi *et al.*, 1992), the United States (Vincelli 1992, Delwiche *et al.*, 1987), Australia (Abbo and Irwin, 1990; Greenhalgh and Merriman, 1988), and New Zealand (Chan and Close, 1987). Lucerne is an important pastoral crop in many countries, and measures to control diseases by *Aphanomyces* sp have focused mainly on the use of fungicides and cropping practices (Payne and Williams, 1990, Papavizas and Ayers, 1974). *A. euteiches* infection is favoured by temperatures in the 22-28°C range and depends on high soil moisture for initiation and spread (Papavizas and Ayers, 1974). There can be considerable variation within the species with respect to host-specificity: a pea pathogen isolate *A. euteiches* f.sp. *pisi* infects peas and beans, whereas *A. euteiches* f.sp. *phaseoli* infects beans but not peas (Pfender and Hagedorn, 1982; Delwiche *et al.*, 1987; Schmitthenner, 1964). *A. euteiches* ICMP 6478, a pea pathogen, was used in this study as the test organism for *in vitro* assays, due to its faster growth at 25°C compared with other fungal pathogens and its ability to grow on most rich culture media.

In this study, a fluorescent pseudomonad obtained from soil was identified as *Pseudomonas aureofaciens* (PA147). This strain was shown to produce an antifungal compound that inhibited the growth of a number of fungal phytopathogens on phosphate buffered potato dextrose agar (PBPDA). To

contribute to the potential use of this strain for biological control, PA147 was chosen for a study of the genetic elements involved in the production of this antifungal compound. This chapter reports the isolation of Tn5 mutants of the wild-type deficient in fungal inhibition of *A. euteiches* on PBPDA, construction of a genomic library, isolation of complementing cosmids and the identification of a locus involved in fungal inhibition. One antibiotic-deficient mutant, PA109, became the subject of further experiments, reported later in this thesis, involving mutagenesis, HPLC, DNA sequencing and *in vivo* glasshouse experiments, to determine a role for wild-type antibiotic production in biological control.

2.1 MATERIALS AND METHODS

2.2 Media, strains and plasmids

A fluorescent pseudomonad isolated by Dr F.R. Sanderson (Crop & Food Research Ltd, Lincoln), designated PA147, was identified as *P. aureofaciens*. A spontaneous rifampicin-resistant derivative, PA147-2, was used for further study. Bacterial strains and plasmids used in this study are shown in Table 2.1. *Escherichia coli* was grown at 37°C on Luria-Bertani (LB) broth or plates (Sambrook *et al.*, 1989). The phytopathogenic fungi used in this study are shown in Table 2.2. Phosphate Buffered Potato Dextrose Agar (PBPDA) was made by boiling 300g potato/per litre of distilled H_2O for 30 mins. The mixture was homogenised and filtered through cheesecloth. Agar (1.5%) was added to the potato filtrate (300ml/l) prior to autoclaving. Sterile glucose (1.0%) and potassium phosphate buffer (pH 6.1, final conc 20 mM) were added after autoclaving. Fungal strains were maintained on PBPDA at 25°C with periodic growth on water agar or PBPDA supplemented with tetracycline and streptomycin, to prevent bacterial contamination. *Pseudomonas* isolates were grown at 30°C on Kings B Medium (KBM) (King *et al.*, 1954) or LB agar. Unless otherwise stated, antibiotics and reagents were used at the following concentrations (mg/ml): rifampicin 50; kanamycin 50; ampicillin 100;

tetracycline 15; streptomycin 20; chloramphenicol 30; gentamycin 50; 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (Xgal) 25.

2.3 Fungal Inhibition Assay

PA147-2 was tested for inhibition of plant pathogenic fungi on PBPDA (Table 2.2). Potassium phosphate buffer (20 mM) was included to maintain the media at pH 6.1-6.8, alleviating acidification of the media in the zone of inhibition caused by PA147-2 during growth. Bacterial colonies were streaked on the edge of a PBPDA plate, which was inoculated at the centre with a 1.0-cm-diameter plug of actively growing fungus (obtained from Crop & Food Research Ltd, Lincoln). The plates were incubated at 25°C for 3-4 days then scored for inhibition of fungal growth.

2.4 Tn5 mutagenesis of PA147-2 and isolation of mutants

Antifungal deficient (Af^-) mutants of PA147-2 were generated using the Tn5 delivery system of Simon *et al.,* (1983). The cells of the donor *E. coli* S17 harbouring the suicide plasmid pSUP2021, were conjugated with the recipient, PA147-2. Overnight broths of donor (pSUP2021) and recipient (PA147-2) were mixed (donor-recipient ratio of 1:1) and 0.5 ml pipetted onto a 0.22-mm-pore-size Millipore filter on a prewarmed LB agar plate. The bacteria were incubated at 30°C for 12-16h, then the conjugation mix scraped from the filters and resuspended in LB. Serial dilutions were spread on KBM plates supplemented with rifampicin (to select for the recipient) and kanamycin (to select for transposition of Tn5). Transconjugants were observed after growth at 30°C for 48h. Purified transconjugants were screened for loss of antifungal activity (Af^-) using the fungal inhibition assay.

2.5 DNA Manipulations

Extraction of plasmid DNA from *E. coli*, preparation of competent cells, ligations and transformations were performed using standard methods (Sambrook *et al.*, 1989, Rodriguez and Tait, 1983). Recombinant plasmids were extracted from *Pseudomonas* by the method of Kado and Liu (1981). Restriction digests were performed according to the manufacturer's recommendations (BRL, Boehringer Mannheim, NEB). Genomic DNA was extracted from *Pseudomonas* and *E. coli* by standard methods (Ausubel *et al*, 1990).

2.6 DNA-DNA hybridisations

DNA probes for Southern analysis were labelled with ^{32}P-deoxynucleotide triphosphates and prepared by the multiprime DNA labelling system (Amersham) according to the manufacturer's recommendations. Southern analysis and colony hybridisations on nylon membranes (HyBond N^+) were carried out according to the manufacturer's instructions however, filters for colony screening were washed prior to hybridisation in 3xSSC (0.45M NaCl, 0.045M sodium citrate, 0.1% sodium dodecyl sulphate) for 3h at 65^{o}C, to remove cellular debris.

2.7 Sucrose gradient fractionation

Genomic DNA (100 mg) from PA147-2 was partially digested with *Sau*3A and fractionated on a 10-40% sucrose gradient, by centrifugation at 20,000 rpm, 18h at 20^{o}C in a Beckman SW41 rotor. Aliquots (1ml) were collected from the gradients and analysed by electrophoresis on 0.4% agarose gels for 20h at 20^{o}C. Fractions with DNA fragments ranging from 15 to 30kb were pooled and used to construct a genomic library.

2.8 Genomic library construction

Size fractionated *Sau*3A-digested genomic DNA of PA147-2 (200 mg/ml) was ligated (BRL Ligase 50U/ml) to pLAFR3 (100 mg/ml) digested with *Bam*HI. Prior to ligation with PA147-2 DNA, *Bam*HI digested pLAFR3 was treated with calf intestine phosphatase (Boehringer Mannheim) and controls for this treatment showed that 98% of linearised vector was inhibited for self ligation. PA147-2 insert and pLAFR3 vector DNA was ligated at 2:1 insert:vector ratio in a volume of 10 µl at room temperature for 3 hours (Boehringer Mannheim). Ligated DNA was packaged in Lambda phage heads using the Packagene Lambda DNA packaging system (Promega) and transduced into *E. coli* DH5α. After incubation at 37°C for 1h, cells were spread on LB agar supplemented with tetracycline and X-Gal. Following overnight incubation at 37°C, plates were scored for white colonies. Plasmid minipreps of 50 randomly picked library colonies followed by double digestion with *Eco*RI and *Bam*HI showed 90% of the cosmids carried genomic insert DNA (data not shown). The library was titered (3.4 x 10^6), amplified and stored at -80°C in 80% sterile glycerol according to standard methods (Sambrook *et al.*, 1989).

2.9 Complementation analysis by allele-replacement

The genomic library was maintained on replica plates (LB agar supplemented with tetracycline and X-Gal) at 4°C. Complementing cosmids were isolated by colony hybridisation, using a region of DNA flanking the Tn5 insertion in the Af$^-$ mutants as a ^{32}P-DNA probe. Putative complementing cosmids were transferred into the Af- mutants by triparental mating with the helper plasmid pRK2013. Aliquots (500 ml) of overnight cultures of *E. coli* HB101(pRK2013), Af$^-$ mutant and the putative complementing cosmid were mixed (1:1:1 ratio) and the cells concentrated by centrifugation. Cells were resuspended (300 ml of fresh LB),

pipetted onto a Millipore filter (0.22 mm-pore-size) on a prewarmed LB agar plate, and incubated for 12-16h at 30°C. Bacteria were scraped from the filters and resuspended in LB. Serial dilutions (0.1ml) were spread on LB containing rifampicin, kanamycin and tetracycline. After 48h at 30°C, transconjugants were pooled and inoculated into 100ml LB broth containing only rifampicin and grown for 12h at 30°C, with vigorous shaking. Serial dilutions were plated onto LB plates containing rifampicin and incubated at 30°C until colonies appeared. Colonies were screened for rifampicin resistance, kanamycin sensitivity (loss of Tn5) and tetracycline sensitivity (loss of pLAFR3), to indicate an allele-replacement event. Recombinant mutants created by allele-replacement were bioassayed for restoration of fungal inhibition (Af^+) on PBPDA.

TABLE 2.1 Bacterial strains and Plasmids[a]

Strain	Relevant Characteristics	Source
E. coli		
S-17	C600, recA	
	[RP4-2-Tc::Mu] *thi, thr, leu, sulII*, Km^r	Simon *et al.*, 1983
HB101	recAB *hsdR hsdM* Str^r*pro leu thi*, F^-	Boyer *et al.*, 1969[1]
DH5α	F^- *endA1 hsdR17 supE44 thi-1*	
	gyrA96 relA1 (argF *lacZYA*)	Sambrook *et al*, 1989
P. aureofaciens		
PA147	wild-type, Af^+	This study
PA147-2	wild-type Rif^R (spontaneous), Af^+	"
PA1	PA147-2::Tn5, Af^-	"
PA109	PA147-2::Tn5, Af^-	"

PA111	PA147-2::Tn5, Af^-	"
PA138	PA147-2::Tn5, Af^-	"
PA30	PA147-2::Tn5, Af^-	"
PA35	PA147-2::Tn5, Af^-	"
PA3	PA147-2::Tn5, Af^-	"
PA17	PA147-2::Tn5, Af^-	"
PA57	PA147-2::Tn5, Af^-	"
PA21	PA147-2::Tn5, Af^-	"
PA95	PA147-2::Tn5, Af^-	"
PA75	PA147-2::Tn5, Af^-	"
PA26	PA147-2::Tn5, Af^-	"
PA109R-102	PA109-allele-replacement mutant, Af^+	"
PA109R-186	PA109-allele-replacement mutant, Af^+	"

Plasmid	Characteristics	Source
pACYC184	Cm^r Tet^r	Chang *et al.*, 1978[2]
pBR322	Ap^r Tet^r	Bolivar *et al.*, 1977
pRK2013	IncP4 Tra^+ Mob^+ Km^r	Ditta *et al.*, 1980
pFC109	16.0 kb::Tn5 *Eco*RI in pBR322 Ap^r Tet^r Km^r	This study
pFC1	16.0 kb::Tn5 *Eco*RI in pBR322 Ap^r Tet^r Km^r	"
pFC111	16.0 kb::Tn5 *Eco*RI in pBR322 Ap^r Tet^r Km^r	"
pFC138	13.5 kb::Tn5 *Eco*RI in pBR322 Ap^r Tet^r Km^r	"
pFCA3	12.2 kb::Tn5 *Eco*RI in pBR322	"

	Ap^r Tet^r Km^r	
pFC35	5.0 kb::Tn5 *Eco*RI in pBR322	"
	Ap^r Tet^r Km^r	
pFC30	12.5 kb::Tn5 *Eco*RI in pBR322	"
	Ap^r Tet^r Km^r	
pFC26	8.0 kb::Tn5 *Eco*RI in pBR322	"
	Ap^r Tet^r Km^r	
pFC916	16.0 kb *Eco*RI from pPS7138 in pSup104	"
	Tet^r	
pFC900	16.0 kb *Eco*RI in pACYC1 Tet^r	This study
pFC900B	16.0 kb *Eco*RI in pBR322, Amp^r, Tet^r	"
pLAFR3	IncP Tet^r cos	Stastkawicz, *et al.*,1987
pSup104	Cm^r Tet^r	Priefer *et al.*, 1985
pSup2021	Cm^r Nm-Km^r Ap^r Mob^+ Tc::Tn5	Simon *et al.*, 1983
pPS7138	pLAFR3 containing PA147-2	This study
	genomic DNA, Tet^r	
pPS265	" "	"
pPS259	" "	"
pPS8108	" "	"
pPS5105	" "	"
pMNU	pBR322::Tn5	This laboratory

[a] Af^+ produces antifungal activity; Af^- deficient for antifungal activity

1 Boyer and Roulland-Dussoix, 1969

2 Chang and Cohen, 1979

TABLE 2.2 Pathogenic fungi inhibited by PA147-2

Fungal Culture	Accession No. [b]
OOMYCETES	
Aphanomyces euteiches	ICMP 6478
Phytophthora megasperma var *sojae*	NZ006
DEUTEROMYCETES	
Ascochtya pisi	ICMP 6893
Phoma medicaginis	ICMP 6601
Rhizoctonia solani	NZ 8931
Fusarium oxysporum	NZ 8870
Fusarium solani	ICMP 8578
ASCOMYCETES	
Mycosphaerella pinodes	ICMP 2268
Gaeumannomyces graminis var *tritici*	NZ8a

[b] ICMP; International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand.

2.10 RESULTS

2.11 Characterisation of a fluorescent pseudomonad with antifungal activity

Pseudomonas aureofaciens PA147 is naturally resistant to ampicillin, and was isolated from soil in the Canterbury area (Crop & Food Research Ltd, Lincoln, NZ). This strain was identified as *P. aureofaciens* using API 20 NE strips (98% identification), and produces the characteristic yellow-green fluorescent siderophores on KBM (Fig 2.1). However, it does not produce the pigmented phenazines characteristic of *P. aureofaciens* (Turner and Messenger, 1986) or hydrogen cyanide (HCN) (Voisard *et al.*, 1989). Furthermore, *P. aureofaciens* 147 has a single polar flagellum (data not shown), whereas *P. aureofaciens* has been characterised with several polar flagella (Stanier *et al.*, 1966). A spontaneous rifampicin-resistant mutant of PA147 was isolated and designated PA147-2. Plasmids were not detected in PA147-2 using standard plasmid extraction protocols (Kado and Liu, 1981, Sambrook *et al.*, 1989). PA147-2 showed a broad spectrum of antifungal activity against the phytopathogenic fungi shown in Table 2.2.

Antifungal activity of *A. euteiches* and *P. megasperma* was observed on an iron-sufficient medium (PBPDA), suggesting that inhibition is due to the production of at least one antifungal compound rather than iron-limitation by siderophores (Fig 2.2). Since PA147-2 caused acidification of PDA and optimum growth of *A. euteiches* was observed between pH 5.5 to 8, it was necessary to buffer the medium to make a valid assessment of fungal inhibition due to antibiosis. PBPDA was routinely used to assay fungal inhibition as the inclusion of phosphate buffer (pH 6.1-6.5) in the media did not superficially alter the ability of PA147-2 to inhibit *A. euteiches* or other fungal pathogens (Fig 2.2).

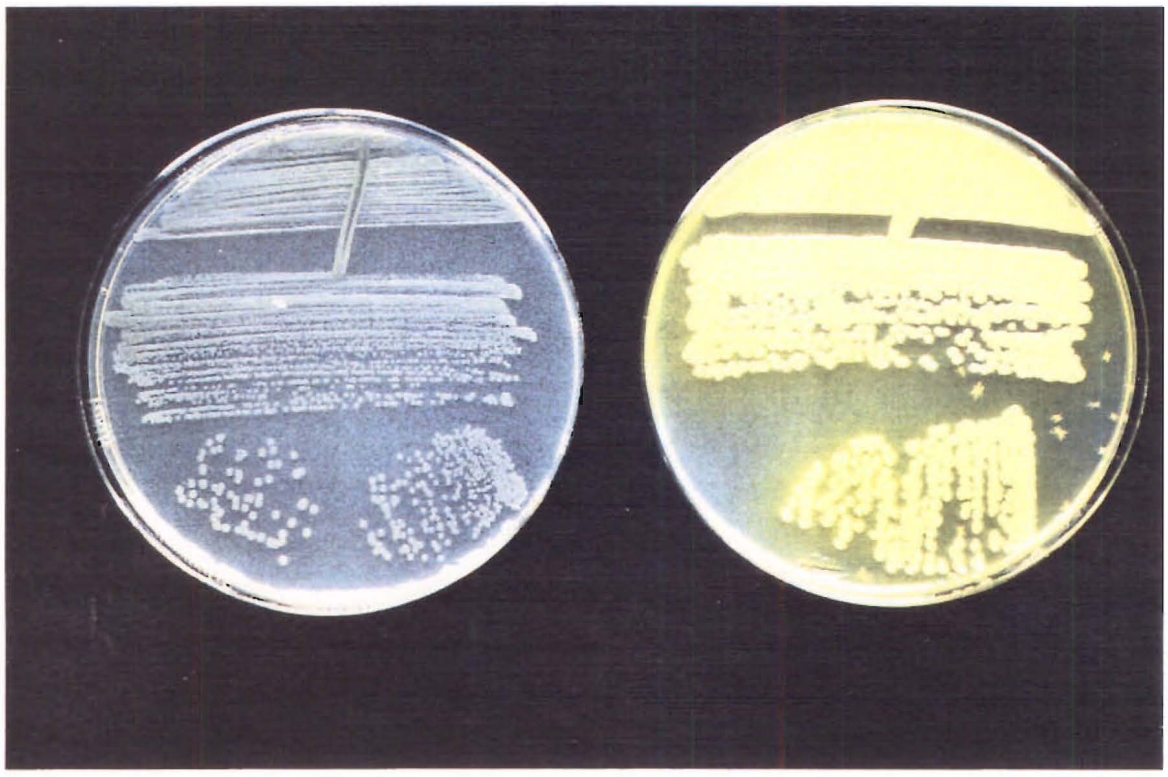
Figure 2.1

A Siderophore production, indicated by yellow colouration in the media, by *P. aureofaciens* strain, PA147-2 on KBM (right) and a non-fluorescent pseudomonad (left), under normal light.

B Fluorescence emitted by PA147-2 under short wave UV light, which is indicative of siderophore production.

Figure 2.1

A



B

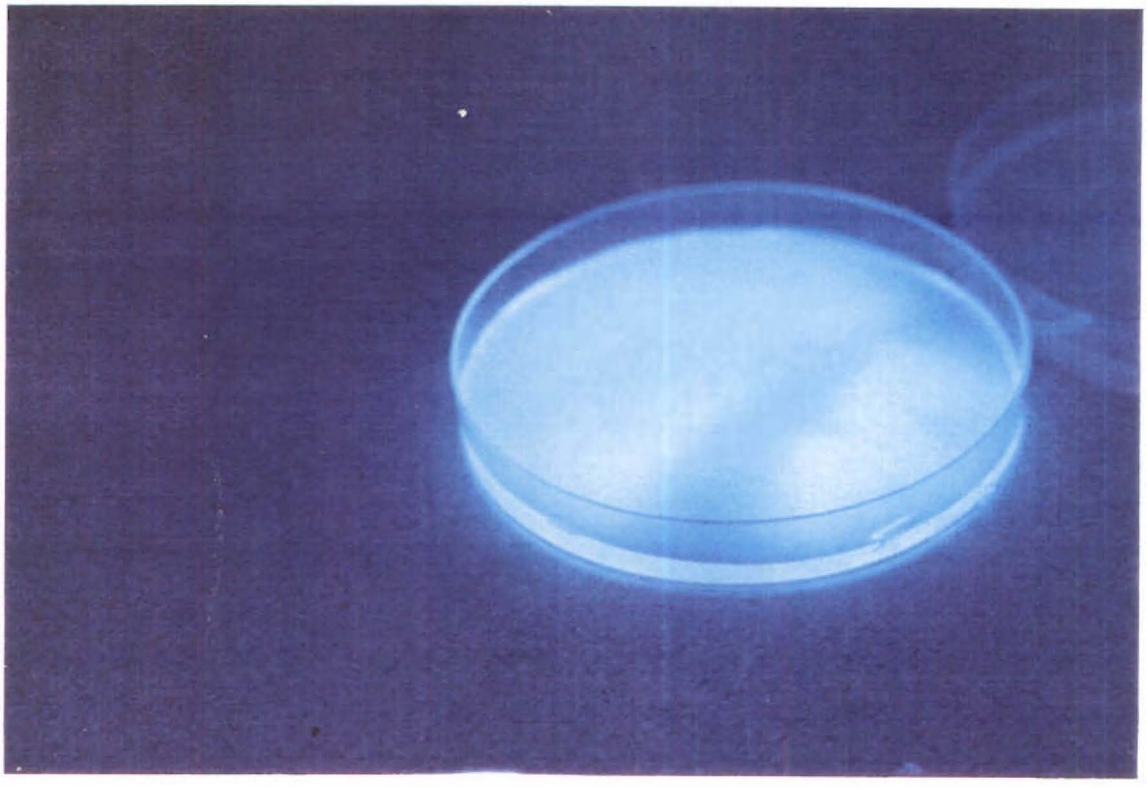


Figure 2.2

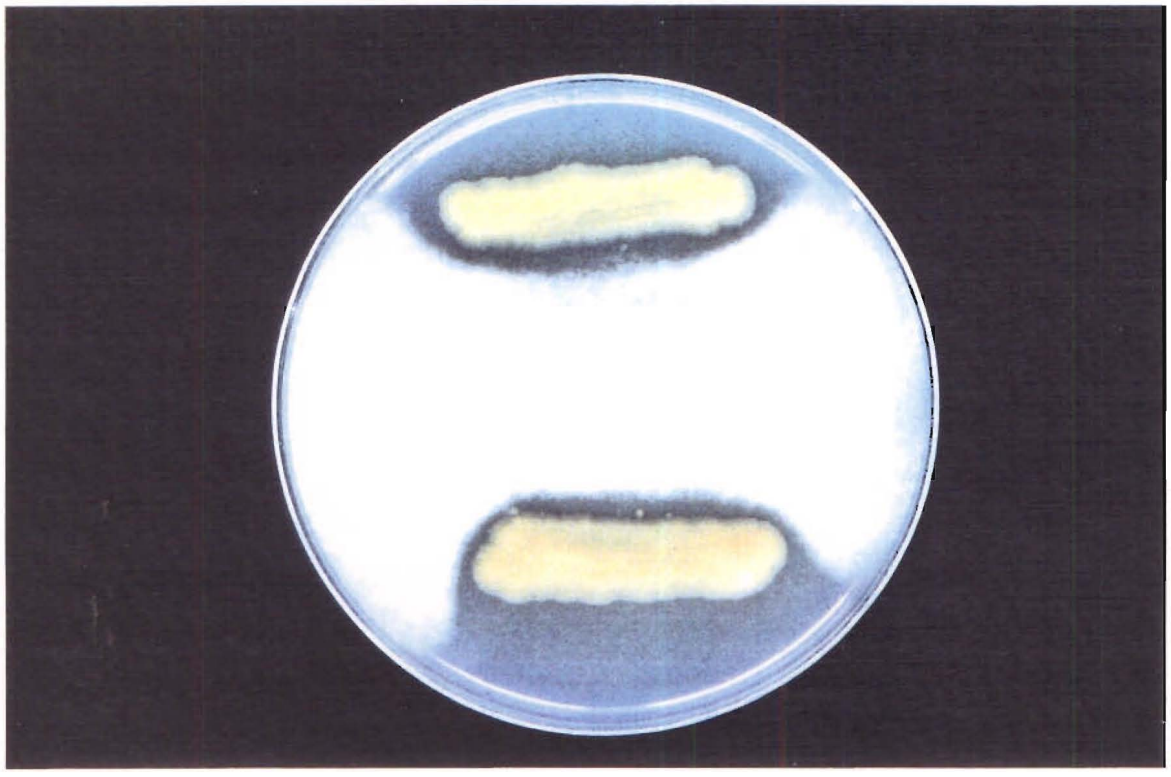
Fungal inhibition assay on iron-sufficient medium, PBPDA, by *P. aureofaciens* strain PA147-2:

A Inhibition of *Aphanomyces euteiches*

B Inhibition of *Phytophthora megasperma* var. *sojae*

Figure 2.2

A



B

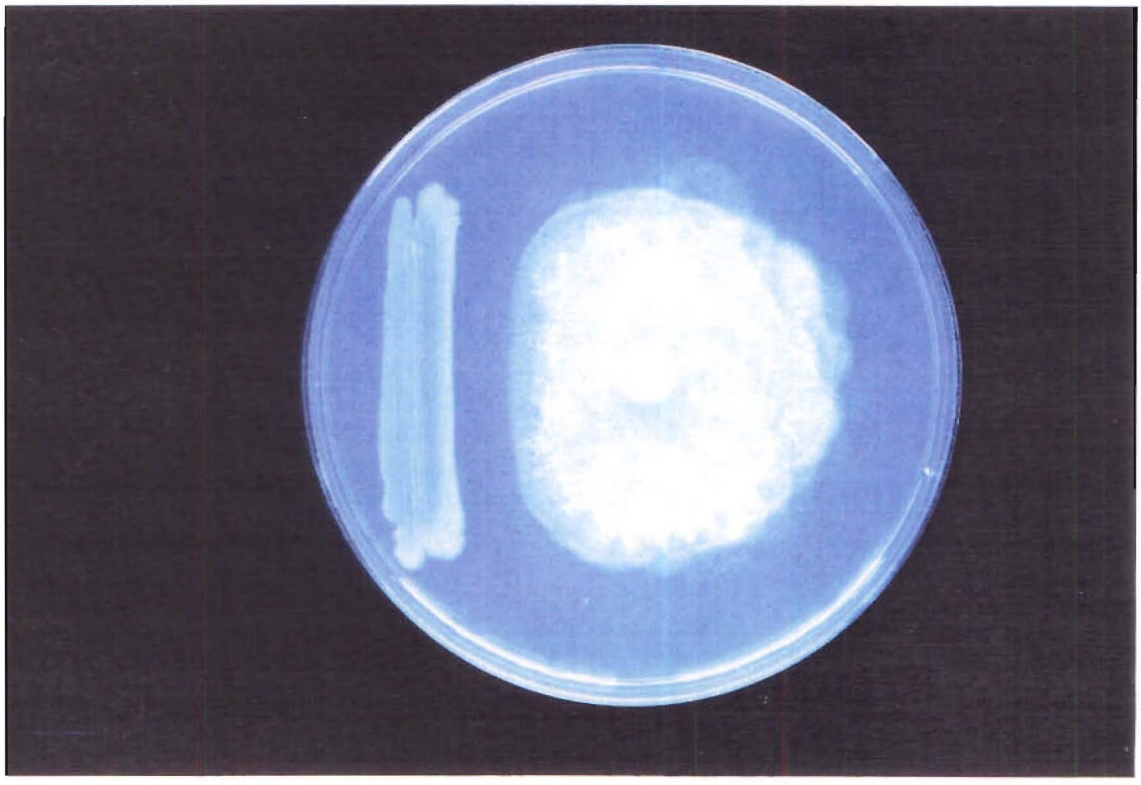


Figure 2.3

Selection of Tn5 mutants deficient for antifungal activity (Af^-)

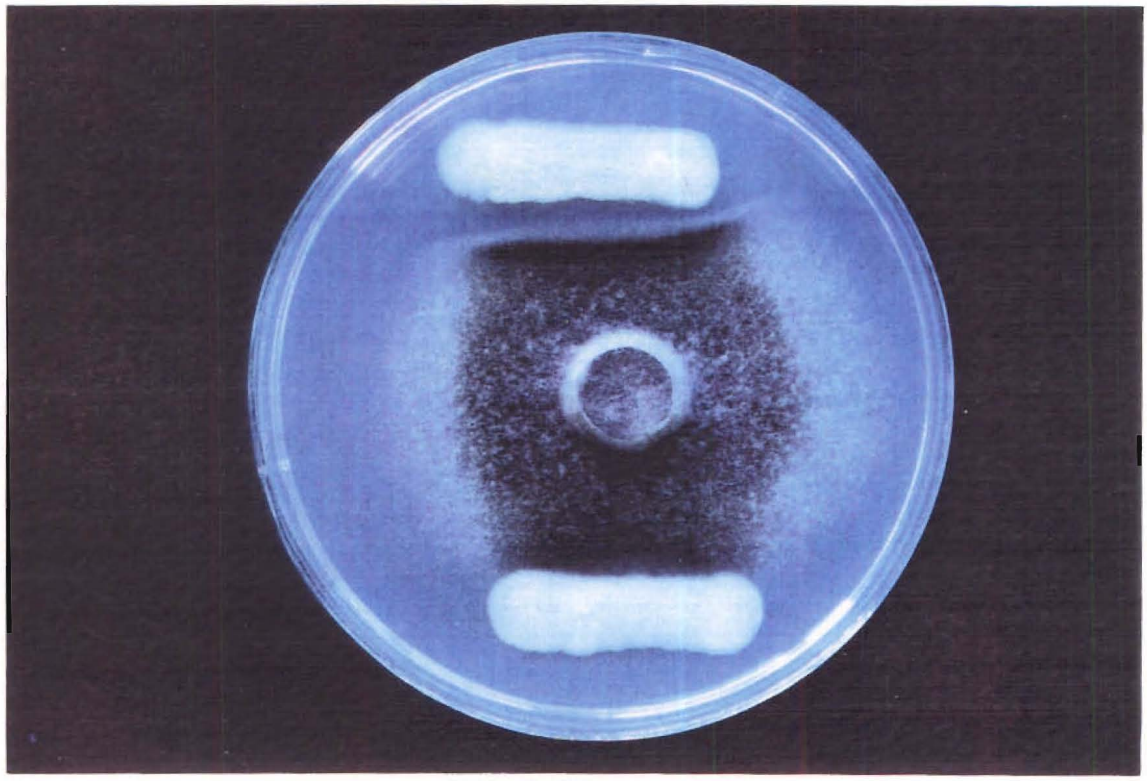
A Inhibition of *A. euteiches*. Af- mutant PA109 (arrow), wild-type Af^+ (c) and remaining colonies not arrowed, PA147-2::Tn5 (Af^+).

B PA147-2 (top) inhibition of *Gaeumannomyces graminis* var *tritici* and Af^- mutant PA109 (bottom).

Figure 2.3

A

B



2.12 Isolation and cloning of antifungal mutants

Mutants defective in antifungal activity (Af^-) were generated by Tn5 mutagenesis. Rifampicin-resistant and kanamycin-resistant colonies (were screened for Af^- against *A. euteiches* on PBPDA (Fig 2.3). Af^- mutants were isolated at a frequency of 2.1×10^{-5} per recipient. Of the total number of transconjugants screened for an Af^- phenotype (1100 transconjugants) approximately 2% were defecifent for fungal inhibition on PBPDA. Spontaneous resistance to kanamycin was less than 10^{-9} and auxotrophic mutants were recovered at a frequency of 1.1%, indicating Tn5 inserts randomly into the chromosome of PA147-2. Thirteen Af^- mutants (Table 2.1) isolated from independent conjugations were unable to inhibit the growth of *A. euteiches* on PBPDA. All Af^- mutants produced siderophores on KBM (data not shown). Mutants PA30 and PA35 were auxotrophic on minimal media and were analysed by DNA sequencing which identified their specific amino acid deficiencies (see Chapter 6). PA1, PA109, PA111, PA138, PA3 and PA21 were Af^- against *G. graminis* var. *tritici*, *Fusarium* spp. and *Phytophthora megasperma* as well as *A. euteiches*. However these six mutants retained antifungal activity against *Mycosphaerella pinodes* and *Ascochyta pisi* on PBPDA (data not shown).

A defined minimal medium developed for the growth of *A. euteiches* (Yang and Schoulties, 1972) was tested as a potential medium for bioassay of PA147-2 mutants, and for further mutagenesis experiments (data not shown). The medium was amended with iron (final concentration 100 mm $FeCl_3$) and phosphate buffer (final concentration 2 mM) to approximate PBPDA conditions. However, PA147-2 and all thirteen Af^- mutants inhibited *A. euteiches*, and despite screening over 1000 Tn5 transconjugants for loss of antifungal activity, no new Af^- mutants were isolated on this media. Typically, the only mutants isolated exhibited poor growth

performance compared to the wild-type or were auxotrophic. This minimal medium may have been too stringent to select for mutations in antibiotic synthesis genes, particularly if the antibiotic precursors are supplied from essential amino acid biosynthetic pathways. The observation that all mutants were Af^- on PBPDA, but Af^+ on amended Yang and Schoulties media, also suggests PA147-2 produces a number of antifungal compounds and that growth conditions are influential on antibiotic production.

To confirm that kanamycin resistance was due to Tn5 insertion, genomic DNA was isolated from each Af^- mutant and digested with *Eco*RI (there are no sites for this restriction enzyme in Tn5). Southern hybridisation of the digested DNA, probed with Tn5, shows a single Tn5 insertion in each mutant (Fig 2.4) and there was no hybridisation between the wild-type DNA and the Tn5 probe (Fig 2.4, lane 1). At least ten unique *Eco*RI fragments contain Tn5 insertions affecting *in vitro* antifungal activity. Mutants PA1, PA109 and PA111 have a Tn5 insertion in the same 16-kb *Eco*RI fragment (Fig 2.4, lanes 2-4). To map the exact position of this Tn5 insertion, the *Eco*RI::Tn5 fragments were cloned from each mutant into the vector pBR322, generating the clones pFC1 (from PA1), pFC109 (from PA109) and pFC111 (from PA111). The restriction pattern for pFC111 was identical to pFC109, therefore PA111 was not analysed further. Restriction enzyme analysis showed the Tn5 insertion was 2.1kb apart in pFC1 and pFC109, indicating mutants PA1 and PA109 have distinct Tn5 insertions, in the same *Eco*RI fragment (Fig 2.5). Tn5-containing inserts were also cloned from mutants PA138 (pFC138), PA30 (pFC30) PA35 (pFC35), PA3 (pFC3) and PA26 (pFC26). Restriction maps for all clones are shown in Figure 2.5.

Figure 2.4

Southern hybridisation of Tn5 insertion in Af$^-$ mutants. Genomic DNA was digested with *Eco*RI and probed with a 2.7-kb *Hind*III internal Tn5 fragment (pMNU). Lanes: 1, PA147-2; Lane 2, PA1; Lane 3, PA109; Lane 4, PA111; Lane 5, PA138; Lane 6, PA95; Lane 7, PA75; Lane 8, PA57; Lane 9, PA17; Lane 10, PA3; Lane 11, PA21; Lane 12, PA30; Lane 13, PA35; Lane 14, PA26. Molecular sizes are shown in kilo bases. An arrow indicates the 16-kb *Eco*RI fragment in lanes 2-4.

Figure 2.4

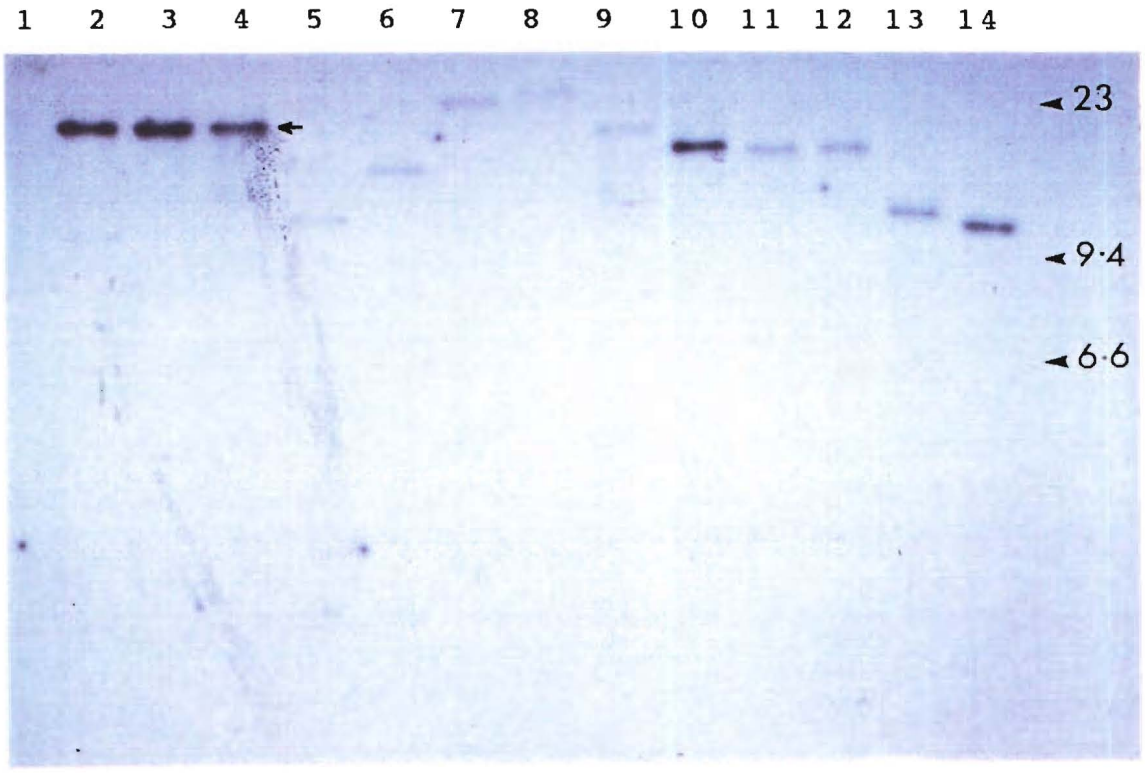


Figure 2.5

Restriction maps of Tn5 insertion fragments cloned from eight Af$^-$ mutants. pFC1 and pFC109 show different Tn5 insertions in the same 16.0 kb *Eco*RI fragment. The arrow indicates the position of Tn5 in each fragment. All *Eco*RI::Tn5 fragments are cloned into pBR322.

RI = *Eco*RI, B = *Bam*HI, Bg = *Bgl*II, S = *Sac*I, H = *Hind*III, X = *Xho*I

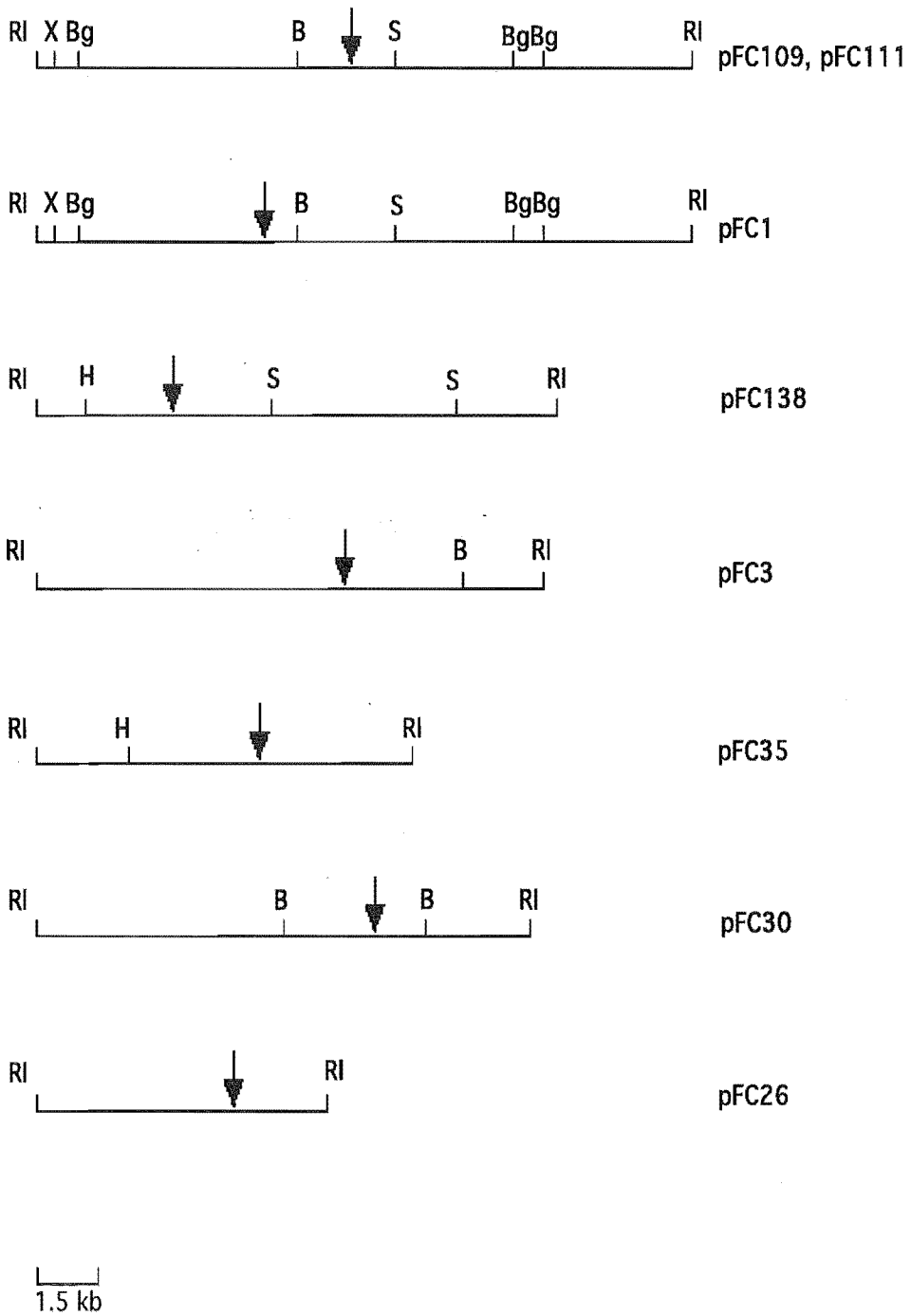
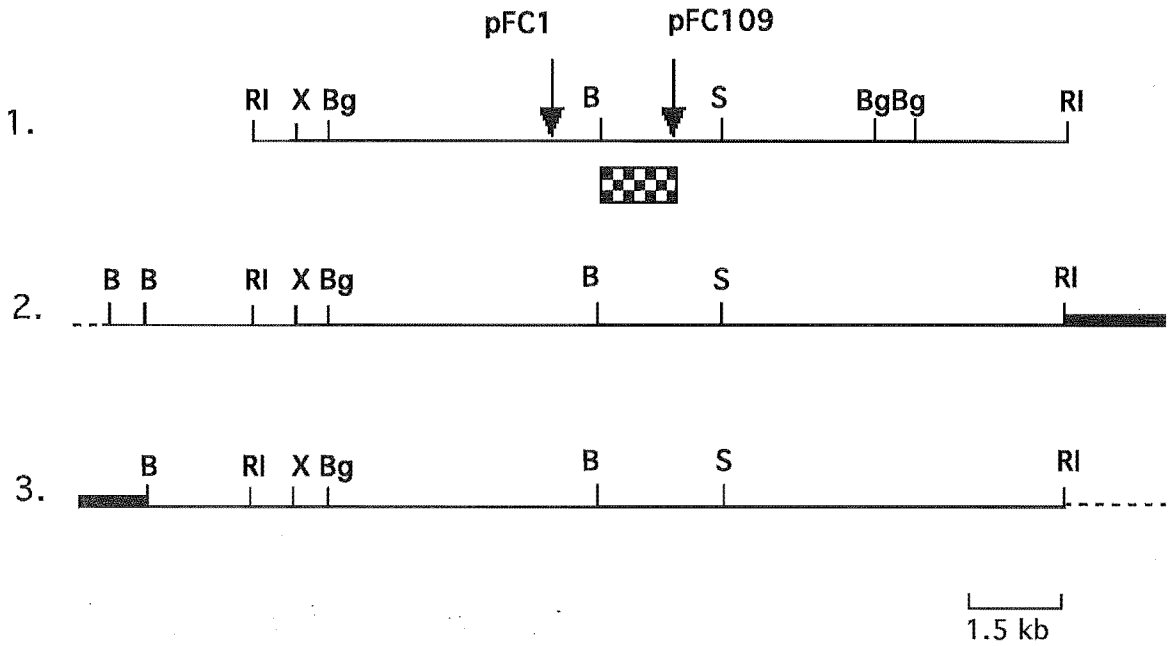

RI X Bg B S BgBg RI
pFC109, pFC111
RI X Bg B S BgBg RI
pFC1
RI H S S RI
pFC138
RI B RI
pFC3
RI H RI
pFC35
RI B B RI
pFC30
RI RI
pFC26
1.5 kb

Figure 2.6



Alignment of the Tn5 regions in PA1 and PA109 with library cosmids.

1. Restriction map of the 16-kb *Eco*RI fragment containing a Tn5 insertion in pFC1 and pFC109. The two clones represent mutants PA1 and PA109, respectively. The arrow indicates the position of Tn5 insertion.

2. Restriction map of cosmids pPS7138 and pPS265.

3. Restriction map of cosmids pPS8108, pPS5105 and pPS259.

In 2 and 3 the dashed line and solid box indicate extra cosmid DNA and cosmid vector, respectively. Checked box indicates a 2.5 kb *Bam*HI-*Hind*III Tn5 flanking fragment from pFC109 used to probe the PA147-2 genomic library (not drawn to scale).

RI = *Eco*RI, B = *Bam*HI, Bg = *Bgl*II, S = *Sac*I, X = *Xho*I

2.13 Complementation by homologous recombination

A genomic library of the wild-type PA147-2 was constructed in the cosmid vector pLAFR3. The library of recombinant cosmids ($3.6x10^6$) contained inserts averaging 22-kb in size, with approximately 1200 recombinant cosmids required to represent 99% of the *Pseudomonas* genome (Romling and Tummler, 1991). To isolate cosmids complementary to PA109, the library was screened with a fragment flanking the Tn5 insertion in PA109 as a ^{32}P-labelled DNA probe. The region of DNA directly flanking both sides of the Tn5 insertion contains homology to wild-type genomic sequences in the library, thereby enabling the rescue of complementing cosmids encoding homologous wild-type DNA. PA109 was chosen for this strategy as the site of Tn5 insertion had been mapped with several restriction enzymes. Furthermore, as PA109 and PA1 contained Tn5 insertions in the same *Eco*RI fragment, separated by 2.1-kb, the complementing cosmids recovered for PA109 were also expected to complement the mutation in PA1.

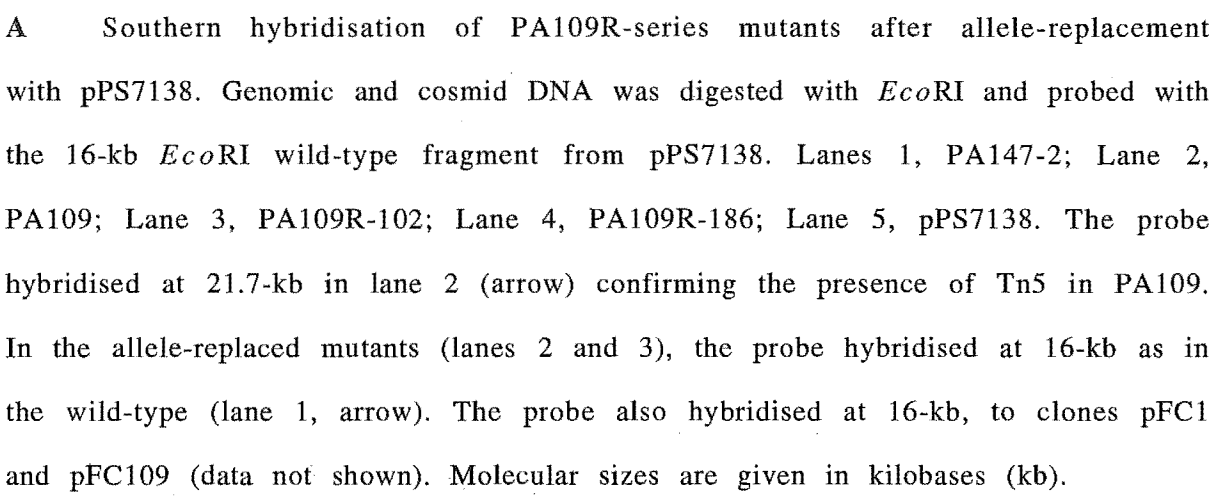
A 2.5 kb *Bam*HI-*Hind*III Tn5-flanking fragment from the clone pFC109 was chosen as the probe to screen the library (Fig 2.6). Seven positive cosmids were isolated with homology to the Tn5 flanking region in PA109. Restriction enzyme analysis identified three cosmids containing the entire 16-kb *Eco*RI fragment, aligned with the restriction maps of pFC109 and pFC1 (Fig 2.6). Each of the seven cosmids were introduced into PA109 by triparental conjugation for complementation analysis. However, plasmid preparations of transconjugants revealed the cosmids were not maintained in the *P. aureofaciens* background without the addition of tetracycline to the medium, and were frequently unstable due to homologous recombination. As *A. euteiches* can not grow on PBPDA supplemented with tetracycline, allele-replacement by homologous recombination was used to replace the Tn5-containing region in PA109, with the homologous wild-type region encoded by the cosmids. Allele replacement between the homologous regions occurred when

pPS7138, pPS265 and pPS259 were introduced into PA109. A number of mutants were obtained by allele-replacement, designated PA109R series, and were sensitive to kanamycin and tetracycline, concomitant with the loss of Tn5 from the chromosome and the cosmid pLAFR3, respectively. The recombined mutants also showed restoration of antifungal activity (Af^+) against *A. euteiches* on PBPDA (data not shown).

2.14 Characterisation of an antifungal locus

A Southern analysis of *Eco*RI digested genomic DNA from two recombined mutants, PA109R-102 and PA109R-186, showed a hybridisation pattern identical to that for PA147-2 (Fig 2.7A; compare lane 1 with lanes 3 and 4). Using the wild-type 16.0 kb *Eco*RI ($Tn5^-$) fragment from pPS7138 as a probe, a 16-kb fragment hybridised in both recombined mutants and corresponded to the same region in PA147-2 (Fig 2.7A; lane 1). This probe also hybridised to the Tn5-containing region in PA109 at 21.7 kb (Fig 2.7A; lane 2). The loss of Tn5 in the recombined mutants restored PA109 to the wild-type hybridisation pattern and to Af^+ on bioassay against *A. euteiches*. The 16-kb *Eco*RI fragment was cloned from pPS7138 into the vector pSUP104 (which is stably maintained in the mutant background without tetracycline selection) to generate the clone pFC916. When pFC916 was tested for complementation *in trans*, it restored PA1 and PA109 to Af^+ against *A. euteiches* on PBPDA. The 16-kb *Eco*RI fragment was also cloned into pACYC184 (pFC900) and pBR322 (pFC900B). A Southern hybridisation was carried out to further confirm origins of the 16-kb *Eco*RI wild-type fragment. Plasmid DNA from pFC900, pF900B, pFC916 and genomic DNA from PA1, PA109 and PA111 was *Eco*RI digested and hybridised with the 16-kb *Eco*RI fragment from pPS7138 (Fig 2.7B). The probe hybridised to the wild-type fragment in lane 1, to the 16-kb *Eco*RI fragment cloned into pFC900, pFC900B and pFC916 (lanes 5-7) and to the homologous Tn5-containing fragments (21.7-kb) in PA1, PA109 and PA111 (lanes 2-4).

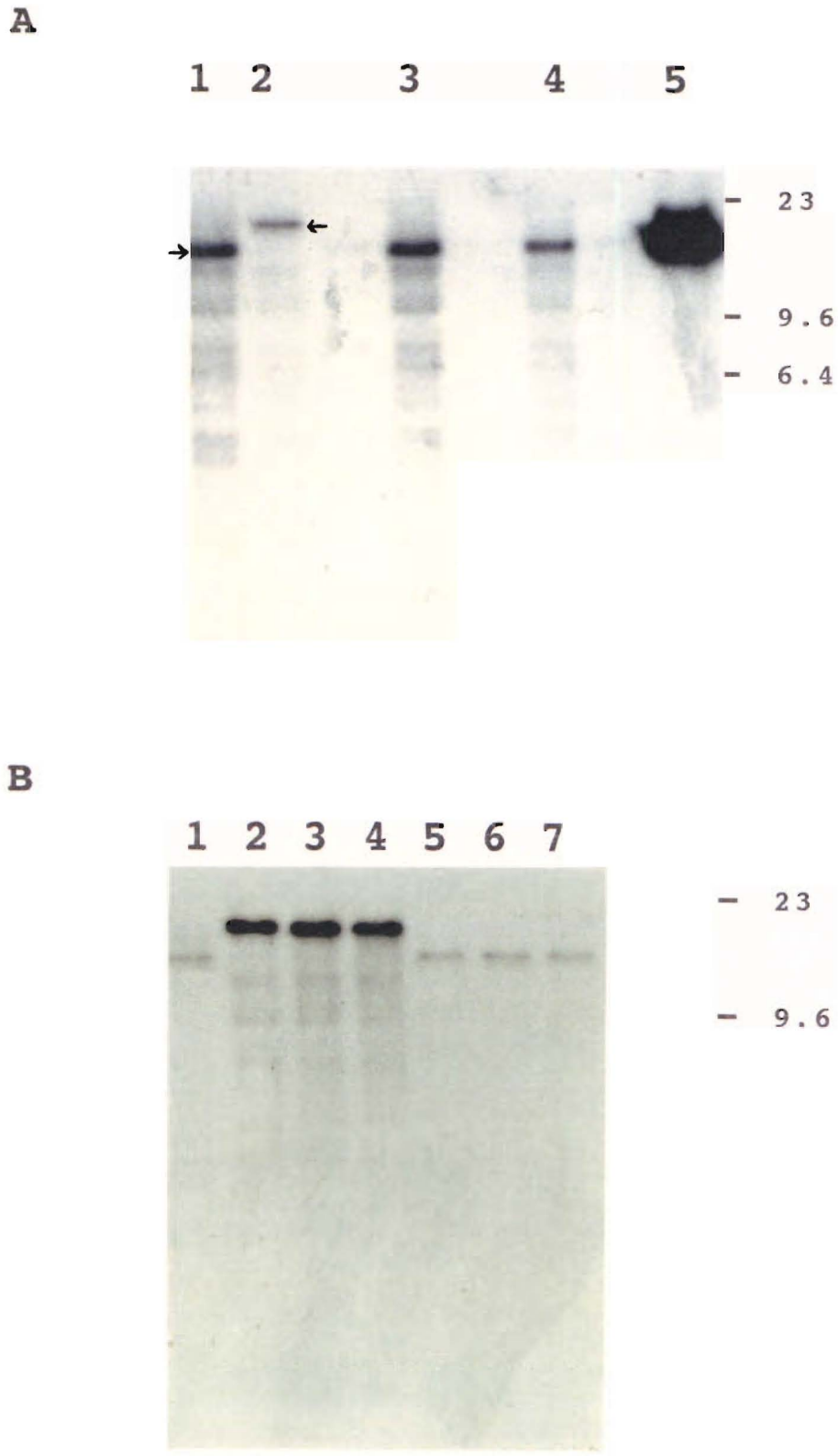
Figure 2.7



A Southern hybridisation of PA109R-series mutants after allele-replacement with pPS7138. Genomic and cosmid DNA was digested with *Eco*RI and probed with the 16-kb *Eco*RI wild-type fragment from pPS7138. Lanes 1, PA147-2; Lane 2, PA109; Lane 3, PA109R-102; Lane 4, PA109R-186; Lane 5, pPS7138. The probe hybridised at 21.7-kb in lane 2 (arrow) confirming the presence of Tn5 in PA109. In the allele-replaced mutants (lanes 2 and 3), the probe hybridised at 16-kb as in the wild-type (lane 1, arrow). The probe also hybridised at 16-kb, to clones pFC1 and pFC109 (data not shown). Molecular sizes are given in kilobases (kb).

B Verification of the cloned 16-kb *Eco*RI region. The probe, 16-kb *Eco*R I fragment from pPS7138, hybridised to *Eco*RI digested PA147-2 genomic DNA (lane 1), to the homologous Tn5-containing fragment in PA1, PA109 and PA111 (lanes 2-4) and to the *Eco*RI cloned insert in pFC900, pFC900B and pFC916 (lanes 5-7 respectively). Molecular sizes are given in kilobases (kb).

Figure 2.7



2.15 DISCUSSION

Characterisation of a *Pseudomonas* strain with antifungal activity

A *Pseudomonas aureofaciens* strain (PA147-2), isolated from soil, inhibits the growth of several major species of phytopathogenic fungi on phosphate buffered PDA. The inhibition of *A. euteiches* on an iron-available medium suggests the observed antifungal activity is due to the production and secretion of at least one antibiotic. Tn5 mutants of PA147-2, deficient in antifungal activity on PBPDA, were restored to wild-type activity through allele-replacement with cosmids from the genomic library, indicating that Tn5 has inserted into loci that are involved in fungal inhibition by production of an antibiotic. Southern hybridisation showed that most of the Af^- mutants have distinct Tn5 insertions in fragments of varying size, suggesting the antibiotic genes are spread throughout the genome. PA1 and PA109, however, were of particular interest because of the position of the Tn5 insertion. These mutants have Tn5 insertions in the same 16.0 kb *Eco*R I fragment, thus it is possible that this locus represents a cluster of genes for both the synthesis and regulation of an antibiotic.

Mutant characterisation

PA147-2 may produce several antifungal compounds with specific activity against certain pathogens. The antibiotic produced by PA147-2 on PBPDA may be specifically active against a group of fungal pathogens. All mutants were Af^- against *A. euteiches*, *G. graminis* var *tritici*, *Fusarium* spp., and *Phytophthora megasperma*, however, six Af^- mutants retained slight inhibition to *M. pinodes* and *A. pisi*. Since PA147-2 normally inhibits *M. pinodes*, the wild-type may produce at least two antibiotics on PBPDA; a major antibiotic that is active against the pathogens *Aphanomyces*, *Fusarium*, *Phytophthora* and *Gaeumannomyces* and

a second, and perhaps minor antibiotic, which has no effect on these pathogens. Tn5 insertion in the six mutants prevents production of the major antibiotic, but may not affect production of the minor compound, which could account for the observed inhibition of *M. pinodes* and *A. pisi*. If the minor antibiotic does not inhibit the fungal pathogens *Aphanomyces, Fusarium, Phytophthora* and *Gaeumannomyces*, any inhibitory activity of the mutants would not be detected against these fungal species on PBPDA. Using HPLC, a single antibiotic was detected that is active against *A. euteiches* (see Chapter 5). However, as the HPLC extracts were not tested against *M. pinodes* it is unclear whether PA147-2 produced an additional *M. pinodes*-specific compound on PBPDA. Production of antibiotics with species-specificity is consistent with results from other pseudomonads. A *P. fluorescens* strain produced two antibiotics, each with distinct activities against *Rhizoctonia solani* and *Pythium ultimum* (Howell and Stipanovic, 1978, 1980). Different antibiotics were also observed when *P. fluorescens* HV37a was grown on media with and without glucose (James and Gutterson, 1986). In addition, a previously undetected antifungal compound, Aff, from *P. fluorescens* 2-79, was found to be a significant factor for inhibition of *G. graminis* var *tritici* (Hamdan *et al.,* 1991). Biological control strains that produced the antibiotic pyrrolnitrin have also been reported to produce other secondary metabolites, such as IAA, that may be significant for disease suppression (Loper and Schroth, 1986). Pyoluteorin, HCN, pyoverdine and an additional antibiotic (Kraus and Loper, 1992), and volatile antifungal compounds (Jayaswal *et al.,* 1992) have been identified from strains of fluorescent pseudomonads. The production and regulation of multiple antibiotics is discussed in Chapter 6.

The biosynthesis of many antibiotics can be strongly dependent on phosphate levels (Martin and Demain *et al.,* 1980), therefore, the inclusion of phosphate buffer to maintain pH of the media could affect antibiotic production and prevent detection of other inhibitory compounds. As strong fungal inhibition was

observed in the phosphate buffered media, the antifungal compound may not be affected by higher phosphate concentration or may be stimulated by higher phosphate levels. An investigation of PA147-2 antibiotic production on a defined medium would help to determine how this antibiotic is regulated and the optimum conditions for production. The conditions for antibiosis on PBPDA may not favour production of other antibiotics by PA147-2. Therefore, some mutants which are Af$^-$ in culture may show disease suppression when tested in soil, if undetectable metabolites contribute to biocontrol *in situ*. This was not the case however, with the one mutant, PA109, assayed for disease control on plants (Chapter 4). *P. fluorescens* strains CHAO and Pf-5 show many similarities to each other in the spectrum of antibiotic production (Kraus and Loper, 1992, Defago and Haas, 1990). Of the antibiotics produced by CHAO and Pf-5, pyoluteorin is most toxic to *Pythium* sp. (Maurhofer *et al.*, 1992).

The cosmid pLAFR3 was unstable in the PA147-2 background without maintenance of tetracycline selection. This has also been observed in other studies (Thomashow and Weller, 1988). In case of PA147-2, it was not possible to assess complementation by individual cosmids *in trans*. To overcome this problem, allele-replacement was used (Ruvkun and Ausubel, 1981) to recombine wild-type sequences from different cosmids into the homologous region of the mutant chromosome. An alternative to allele-replacement is to create *recA*-deficient strains of PA147-2 and Af$^-$ mutants for analysis of complementation *in trans*. The latter approach was recently used to characterise a genetic locus in *P. syringae* pv. *syringae*, involved in production of syringomycin and pathogenesis of bean (Hrabak and Willis, 1992). PA147-2 is resistant to transformation techniques such as chemically-induced competency and electroporation, and does not readily accept all cloning vectors. Despite subcloning the 16 kb wild-type *Eco*RI fragment into pSUP104, a vector that is stable in PA147-2 without antibiotic selection, the assessment of complementation *in trans* was not always reliable and allele-

replacement by homologous recombination was the most successful method to assess mutant complementation. However, allele-replacement prevents detection of cosmids that may partially complement various mutants and may consequently provide less information on the structural and regulatory elements. It is possible that wild-type copies of this region may need to be present *in cis* on the chromosome. It was found that allele-replacement in PA147-2 could be used advantageously to circumvent the difficulties encountered with complementation, subcloning and site-directed mutagenesis (see Chapter 3). The genetic region defined by the 16-kb *Eco*RI fragment in PA1 and PA109, was further analysed and is the focus of work presented in the remainder of this thesis.

CHAPTER 3

Saturation mutagenesis of an antifungal locus

Introduction

Pseudomonas aureofaciens strain PA147-2 shows broad antagonistic activity against a wide range of fungal phytopathogens through the production of at least one major antibiotic under laboratory conditions. Using Tn5 mutagenesis, a genomic region that is involved in the production of an antifungal compound was identified. A 16-kb *Eco*RI region isolated from a genomic library of the wild-type PA147-2 restores antifungal activity in two mutants, PA1 and PA109, deficient in biosynthesis of an antifungal compound. In this chapter a number of miniTn10 mutations were generated specifically in the 16-kb *Eco*RI fragment and recombined into the wild-type chromosome, to assess their effect on fungal inhibition and define the genetic region of antifungal activity.

In this study, the approach to generate mutations in the 16-kb *Eco*RI fragment in the wild-type involved first mutating this region present on a cosmid isolated from the genomic library. The transducing phage λNK1316 (Kleckner *et al.*, 1991) containing a 1.8-kb kanamycin resistant transposon derivative of miniTn10, was used for this mutagenesis approach. This transposon has been designed to prevent multiple insertions of the transposon and therefore reduces the possibility of uncontrolled transposon insertions in the wild-type genome (Kleckner *et al.*, 1991). The desired transposon insertion on the cosmid were then recombined into the wild-type by allele-replacement, following the approach of Ruvkun and Ausubel (1981). The results suggest that this region contains a gene cluster involved in antibiotic production and regulation.

3.1 MATERIALS AND METHODS

3.2 Strains and Plasmids

Bacterial strains and plasmids used in this study are shown in Table 3.1

3.3 Mutagenesis of pPS7138

i) ***Electroporation***

Cosmid pPS7138 was electroporated into *E. coli* W3110 by the following method: LB broth (100 ml) was inoculated with 1 ml of overnight culture of *E. coli* W3110 and grown at 37°C with vigorous shaking to OD_{600} of 0.5 to 0.7. Cells were chilled for 15-30 mins on ice and centrifuged in a Sorvall rotor, JA20 at 4000 rpm for 15 mins at 4°C. Cells were resuspended in 1 volume cold sterile dH_2O and centrifuged as above. Cells were resuspended in 0.5 volume cold dH_2O, centrifuged as above and resuspended in 25 ml dH_2O. After a final centrifugation, cells were resuspended to a final volume of 2-3 ml cold 10% glycerol to a cell concentration of 1-3 x 10^{10} cells/ml. pPS7138 DNA (1-20 ng in TE) was added to 40 ml prepared cells, incubated on ice for 10 min, and transferred to a chilled 0.2cm electroporation cuvette (Bio-Rad). An electroporation pulse was delivered with a field strength approximately 0.5 kV/cm (Gene Pulser at 25 mF and 2.50 kV, Pulse Controller at 200 ohms, time constant between 4-5 msec). SOC medium (1ml) (Sambrook *et al.,* 1989) was added immediately to the cuvette and the electroporated cells incubated at 37°C for 60 min with gentle shaking. Dilutions were plated onto LB supplemented with tetracycline and incubated at 37°C for 12-16 hours.

ii) ***Transduction***

W3110 (pPS7138) purified colonies were grown overnight at 37°C in L-Broth + 0.2% maltose for transduction with λNK1316 (3 x 10^{10} phage/ml). Cells were

concentrated and resuspended in 0.1 volume of LB. W3110 (pPS7138) cells (100 ml) and lNK1316 (multiplicity of infection range 0.2-1.0) were absorbed at room temperature for 15 min, followed by incubation at 37°C for 15 min, then washed twice with 5 ml LB containing sodium citrate (50mM). Cells were concentrated by centrifugation, plated on LB (containing 2.5 mM sodium pyophosphate) supplemented with tetracycline and kanamycin, and incubated overnight at 37°C. Transductants from independent transduction experiments were pooled from each plate in L-Broth and plasmid DNA prepared by standard methods (Sambrook *et al.*, 1989). Plasmid DNA of pooled transductants was electroporated into *E. coli* DH5a (prepared as for W3110), and transformants selected after overnight incubation at 37°C, on LB plates containing tetracycline and kanamycin. Plasmid DNA was prepared from single transformants and the position of the transposon was mapped with restriction enzymes.

3.4 Mutagenesis of PA147-2 by allele-replacement

Each plasmid containing a transposon mutation that mapped to the 16kb *Eco*R I fragment of pPS7138 was introduced into PA147-2 by conjugation. The plasmid pPH1JI, which is incompatible with pPS7138, was then introduced into PA147-2 to force a recombination event between pPS7138 and the homologous region in PA147-2. Triparental conjugations was carried out as follows. Stage 1: Overnight cultures of PA147-2, helper plasmid in *E. coli* HB101 (pRK2013) and individual miniTn10-mutants (pPS7138::miniTn10-kan^r) were mixed and conjugated as previously described (Chapter 2). Transconjugants were selected and purified on LB plates containing rifampicin, ampicillin, kanamycin and tetracycline. Stage 2: Purified transconjugants from stage 1 were conjugated with the incompatible plasmid pPH1JI (*E. coli* HB101) and the helper plasmid pRK2013 (*E. coli* HB101) as described in chapter 2, section 2.3.8. Allele-replaced transconjugants were selected on LB plates containing rifampicin, ampicillin, gentamycin and

kanamycin. Putative recombinants were screened for tetracycline sensitivity, concomitant with loss of pLAFR3. The effect of allele-replaced mutations on the antifungal activity of PA147-2 was tested using the fungal inhibition assay described in Chapter 2.

3.5 DNA-DNA hybridisations

Genomic DNA was extracted from *Pseudomonas* and *E. coli* by standard methods (Sambrook *et al.*, 1989). DNA probes for Southern analysis were labelled with ^{32}P-labelled deoxynucleotide triphosphates and prepared by the Multiprime DNA labelling system (Amersham) according to manufacturer's recommendations. Southern hybridisation on nylon membranes was carried out as previously described in Chapter 2.

TABLE 3.1 Bacterial strains and plasmids

Strain	Characteristics	Source
E. coli		
W3110	wildtype *E. coli*	
DH5α	F⁻ *endA1 hsdR17 supE44 thi-1*	
	gyrA96 relA1 _(arg F⁻ lacZYA)	Sambrook *et al*, 1989
HB101	recAB *hsdR hsdM* Str^r*pro leu thi*, F⁻	Boyer *et al.*, 1969[1]
P. aureofaciens		
PA147-2	wildtype, Rif^r Af$^+$	This study
PA109	PA147-2::Tn5, Rif^r Kan^r Af⁻	"
PA1	PA147-2::Tn5, Rif^r Kan^r Af⁻	"
PA4.2.3	PA147-2::miniTn10, Rif^r Kan^r Af$^+$	"
PA3.2.8	" " Af$^+$	"
PA4.4.17	" " Af⁻	"
PA4.3.6	" " Af⁻	"
PA4.1.2	" " Af⁻	"
PA1.1.1	" " Af⁻	"
PA4.1.4	" " Af⁻	"
Bacteriophage		
λNK1316	l::miniTn10-kan^R	Kleckner *et al.*, 1991

Plasmids	Characteristics	Source
pPS7138	pLAFR3 containing PA147-2 genomic DNA, Tet^r	This study
pRK2013	IncP4 Tra^+ Mob^+ Km^r	Ditta *et al.*, 1980
pPH1JI	tra IncP1 $Gent^r$	Beringer *et al.*, 1978
pPS4.2.3	pPS7138::miniTn10 tet^r kan^r	This study
pPS3.2.8	" "	"
pPS4.4.17	" "	"
pPS4.3.6	" "	"
pPS4.1.2	" "	"
pPS1.1.1	" "	"
pPS4.4.2	" "	"
pPS4.1.4	" "	"

[1] Boyer and Roulland-Dussoix, 1969

3.6 RESULTS

3.7 MiniTn10 mutagenesis of pPS7138

The cosmid pPS7138 contains a 16-kb *Eco*RI wild-type fragment that complements Tn5 mutations in PA1 and PA109. A number of specific transposon mutations were generated in this fragment and then each recombined into the wild-type chromosome. This approach allowed the generation of specific mutations in a defined region on the wild-type chromosome, so that the size of the genomic region involved in antifungal activity on PBPDA could be determined.

The pLAFR3-based cosmid pPS7138 was used with the RK2 replicon-incompatible plasmid pPH1JI (Beringer *et al.,* 1978) for allele-replacement. As described in Chapter 2, cosmid pPS7138 is unstable and is readily lost from the *P. aureofaciens* background in the absence of tetracycline selection. An incompatible plasmid pPH1JI was introduced into transconjugants containing pLAFR3-based miniTn10 constructs, to improve selection for an allele-replacement event between the homologous regions on the wild-type chromosome and the miniTn10 mutations on the cosmid (Ruvkun and Ausubel, 1981). A helper plasmid (pRK2013) was used to aid conjugation as although the pPH1JI plasmid carries its own mobilisation and transfer genes, previous experiments found this plasmid did not transfer efficiently into PA147-2 (data not shown). Recombinant plasmids previously constructed containing the 16-kb *Eco*RI fragment, pFC916, pFC900 and pFC900B, could not be used for allele-replacement. pFC916 was into the cloning vector pSUP104, which is based on the broad-host-range plasmid RSF1010. pSUP104 is both stable in the PA147-2 background and compatible with pH1JI. Furthermore, pFC900 and pFC900B, derived from the cloning vectors pACYC184 and pBR322 respectively, have a narrow host range and do not replicate in PA147-2 (Priefer *et al.,* 1985). To circumvent these problems, the cosmid pPS7138 was used for allele-

replacement. pPS7138 was first mutated with the miniTn10 transposon, λNK1316, in a λ-sensitive, RecA strain of *E. coli*, W3110.

Plasmid DNA was then isolated from pooled transductants and transformed into *recA E. coli* DH5a for further analysis. Eighty four transformants from six independent transductions were mapped with *Eco*RI and *Bam*HI. Ten transposon mutations were chosen for further analysis. Transposon insertions in either a 6.4-kb *Eco*RI-*Bam*HI or 9.6-kb *Bam*HI-*Eco*RI fragment which comprise the 16-kb *Eco*RI fragment (Fig 3.1) were selected. The mutations in pPS4.3.9 and pPS4.2.5 mapped to the same position as pPS4.2.3 and therefore only pPS4.2.3 was used in allele-replacement experiments (data not shown). Similarly, pPS4.4.5 mapped very close to pPS4.4.17 and therefore, pPS4.4.17 was chosen for allele-replacement (Fig 3.1).

3.8 Effect of mutations on antibiotic production by PA147-2

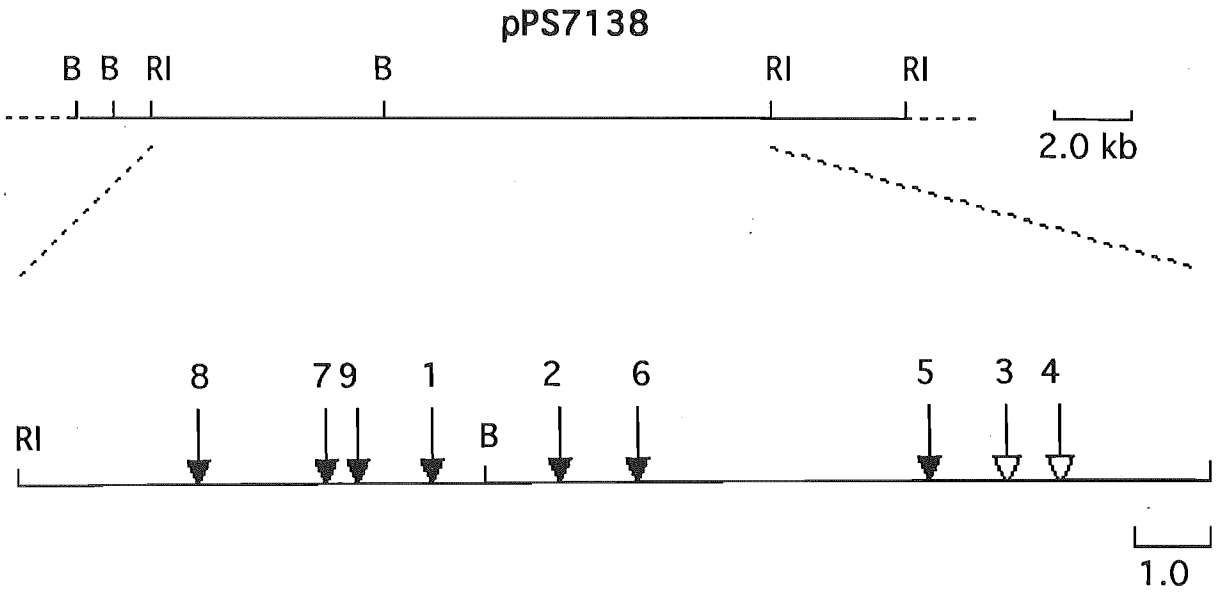
The effect of miniTn10 mutation on antifungal activity by PA147-2 was determined by the fungal inhibition assay and the results are summarised in Fig 3.1. MiniTn10 mutation in PA3.2.8 had no affect on antibiotic production on PBPDA (unfilled arrow, Af^+). Similarly mutant PA4.2.3, with a miniTn10 insertion approximately 500bp from PA3.2.8, also retained inhibitory activity of *A. euteiches* (unfilled arrow, Af^+). Therefore, PA3.2.8 defined one of the boundaries of the antifungal region in this fragment. MiniTn10 mutant PA4.4.17, approximately 1.0 kb from PA3.2.8, no longer inhibited *A. euteiches* (filled arrow, Af^-). Furthermore, mutants PA4.3.6, PA4.1.2, PA4.1.4 and PA1.1.1 are also deficient for antibiotic activity.

FIGURE 3.1

Saturation mutagenesis of pPS7138 with miniTn10-kan^r

MiniTn10-kan^r insertions were mapped to the 16-kb *Eco*RI fragment in pPS7138. Each mutation was recombined into PA147-2 to give rise to stable kan^r mutants, as shown in the Key, numbers 3-10. The site of Tn5 insertion in the 16-kb *Eco*R I fragment of PA1 and PA109 is also shown for reference. The dashed lines from pPS7138 denote cosmid DNA outside the region of interest. Filled arrow = miniTn10-kan^r insertion, Af$^-$. Unfilled arrow = miniTn10-kan^r insertion, Af$^+$. B = *Bam*HI, RI = *Eco*RI

Saturation mutagenesis of pPS7138



KEY to miniTn10-kan mutations in 16-kb *Eco*RI fragment

1. Mutant PA1, Tn5 insertion
2. Mutant PA109, Tn5 insertion
3. PA3.2.8
4. PA4.2.3
5. PA4.4.17
6. PA4.3.6
7. PA1.1.1
8. PA4.1.4
9. PA4.1.2

B=*Bam*HI, RI=EcoRI, dotted line on pPS7138 =cosmid DNA outside region of interest,

▼ mini Tn10 insertion, Af- ▽ miniTn10 insertion, Af+

3.9 DISCUSSION

The results presented in this chapter describe the delineation of one boundary of a genomic region involved in antifungal activity by PA147-2. The Tn5 insertions in two Af$^-$ mutants PA1 and PA109 have been mapped to this region and miniTn10 mutagensis has identified at least 13 kb that is required for fungal inhibition on PBPDA. This region is by no means saturated by a transposon insertion appraoach and further mutagenesis experiments are required to define the boundaries of this region in more detail. It was not possible to isolate a miniTn10 insertion closer than approximately 2 kb to the left hand *Eco*RI site (i.e. proximal to the location of Tn5 in PA1, see Fig 3.1). This may be acheived in subsequent mutagenesis experiments. It is possible that further genes involved in antifungal activity are located beyond the boundary of this 16kb fragment. In a recent study, a novel regulator of 2,4-diacetylphloroglucinol (Phl) and pyoluteorin (Plt) production was identified using a similar approach to that described in this chapter (Schnider *et al.*, 1994). A cosmid isolated from a genomic library of a biological control strain *P. fluorescens* CHAO was subcloned into the vector pVK100 and a 7-kb *Bgl*II-*Hind*III fragment was found to cause over-production of both Phl and Plt. Using the transposon Tn*1737*Cm, a 2.3-kb region was localised, which on its own enhances antibiotic production (Schnider *et al.*, 1994).

The approach to generate site-directed mutations in PA147-2 and determine their effect on antifungal activity was similar to that described for the creation of nitrogen-fixation mutations in *Rhizobium* (Ruvkun and Ausubel, 1981). To circumvent the previous difficulties with recombination and instability of vectors when assessing fragments *in trans* in a PA147-2 background, defined mutations were created in the wild-type for fungal inhibition assays *in vitro* and glasshouse trials. A non-hopping derivative was chosen to ensure that PA147-2 became

kanamycin resistant through allele-replacement and not through random transposition by miniTn10. Two independent conjugations were required for site-directed mutagenesis of PA147-2. Firstly, eight pPS7138::miniTn10-kan^r mutations (Table 3.1) were individually introduced into PA147-2 by tripartite conjugation to give PA147-2 tranconjugants containing the pPS7138::miniTn10-kan^r construct. Here, an additional antibiotic selection, ampicillin, was included to favour the recovery of PA147-2 transconjugants and select against growth of the donor and helper plasmid *E. coli* strains. Secondly, recombination was forced between the homologous regions on the PA147-2 chromosome and the pPS7138::minTn10-kan^r, through the introduction of pH1JI. An allele-replacement event resulted in tetracycline sensitivity (indicating loss of pPS7138) and kanamycin resistance, indicating insertion of miniTn10-kan^r in PA147-2. Seven site-directed miniTn10-kan^r mutants of PA147-2 were generated in this way (Table 3.1). In some cases pH1JI was passively lost when transconjugants were subsequently purified in the absence of gentamycin selection. The presence of pH1JI in the cell did not interfere with antifungal activity, as a control of PA147-2 containing only pH1JI was unaffected in its ability to inhibit *A. euteiches* on PBPDA (data not shown).

CHAPTER 4

The significance of antibiotic production by *Pseudomonas aureofaciens* PA147-2 for biological control of *Phytophthora* root rot of asparagus.

Introduction

There is growing evidence that the production of antibiotics by some biocontrol bacterial strains is an important mechanism for disease suppression (Fravel, 1988). However, *in vitro* production of antibiotics is not always an indication of *in vivo* biocontrol. It is therefore critical to test a putative strain under glasshouse and field conditions to accurately assess its performance. Antibiotic-negative mutants of some biocontrol strains have been used to identify a role for antibiotics such as pyrrolnitrin and pyoluteorin (Homma, 1994, Howell and Stipanovic, 1979, 1980), phenazine-1-carboxylate (Thomashow and Weller, 1988), 2,4-diacetylphloroglucinol (Keel *et al.,* 1992) and oomycin A (Howie and Suslow, 1991) in biological control of fungal pathogens on plants. In a recent study, mutants of *P. fluorescens* CHAO defective in a range of antifungal metabolites were tested for their contribution to the biocontrol ability of this strain (Maurhofer *et al.,* 1994b). The results suggest that metabolites produced by CHAO, which include pyoverdine, salicylate, pyochelin, HCN, pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol and indole acetic acid, vary in their importance to control plant diseases. The use of mutants to study biocontrol mechanisms has helped to identify specific antifungal metabolites which account for disease suppression (Weller, 1988).

Phytophthora megasperma var. *sojae* and *P. crytogea* are important pathogens of asparagus, affecting production in California and New Zealand (Falloon, 1982, Falloon *et al.,* 1986, Falloon and Grogan, 1988). *P. megasperma* var *sojae* is the main

causal agent of crown and root rot of asparagus in New Zealand and is strongly pathogenic to asparagus. The disease can be controlled in wet seasons with Metalaxyl, however, the rate required to maintain disease control would be economically inviable to the industry (Falloon, 1982). The *Phytophthora* pathogen is most severe on asparagus seedlings, as the young crown and root tissue are more susceptible to *Phytophthora* rot than older tissue. Screening for seedlings resistant to the pathogen has been recommended as a means to reduce severity of the disease in the field (Falloon and Grogan, 1991). Therefore, there is great potential for the application of a biocontrol strain to control *Phytophthora* rot and provide an alternative to chemical fungicides.

An antibiotic produced by *P. aureofaciens* PA147-2 appears to be important for the inhibition of a number of fungal pathogens *in vitro* including *P. megasperma* var. *sojae* (Chapter 2). A Tn5 mutant, PA109, does not produce the antibiotic on phosphate buffered potato dextrose agar (PBPDA). The loss of Tn5 by allele-replacement restores PA109 to wild-type activity. In this chapter the role of the antibiotic produced by PA147-2 for the biological control of plant fungal diseases was investigated. To establish whether there was a correlation between *in vitro* antibiotic synthesis and disease suppression *in planta*, the wild-type PA147-2, Af-mutant PA109, and PA109R-186 (PA109 restored to antibiotic activity by complementation), were tested for their ability to suppress *Phytophthora* root rot of asparagus seedlings. These strains were also assessed for their ability to promote plant growth in the absence of the pathogen.

4.1 MATERIALS AND METHODS

4.2 Preparation of fungal and bacterial inoculants

To prepare fungal inoculum, petri dishes containing 20% V-8 Juice agar (Falloon, 1985) were inoculated at their centre with a 5 mm diameter single plug of actively growing fungus (isolate *Phytophthora megasperma* var *sojae* NZ006) and incubated for 9 days at 23°C under an 18 hour photoperiod (10µmol/m^2/sec). The plates were flooded with 20 ml of sterile pond water and incubated a further 6 days. Contents of the agar plates were then homogenised in a blender with 500 ml to 1 litre sterile pond water. *Pseudomonas* strains were grown in 200 ml 20% L-broth for 24 h at 30°C, to an OD_{600} of 1.5 (approximately $1x10^9$ cells/ml), and the bacterial suspensions used for seedling inoculation.

4.3 *In planta* assay for disease suppression

An assay to evaluate protection against *Phytophthora* root rot disease of asparagus by *Pseudomonas* strains was derived from Falloon and Grogan (1991). The asparagus genotype was a hybrid between a female (CRD74) clone and a male (CRD70) clone selected from Mary Washington 500. Five seeds were planted in each pot (6.5 cm^3) containing 3 parts peat:1 part sand, supplemented with 4.8 kg/m^3 dolomite lime, 1.0 kg/m^3 superphosphate, 0.09 kg/m^3 calcium nitrate, 2.5 kg/m^3 osmocote (14-6.1-11.6, 3-4 month; Sierra) 0.04 kg/m^3 Fetrilon Combi 1 (BASF), and 0.75 kg/m^3 Micromax TM (Sierra). Plants were grown in a greenhouse and maintained with heating <18°C and ventilation >22°C until seedling establishment (approximately 21 days), then transferred to a growth room maintained at 15°C with 70% humidity and 16h photoperiod (150 mmol/m^2/sec), to promote development of *Phytophthora* root rot (Falloon and Grogan, 1991). The pots were flooded with tap water by placing them into another container which

was then filled with water to the soil level in the pots. Following flooding, the pots were inoculated with 10 ml bacterial suspension, and 24 hours later, 20 ml fungal suspension, followed by a further 10 ml bacterial suspension 2-3 h following application of fungi. Fungal minus controls received only 20 ml V-8 agar homogenised in sterile pond water. After 2 days, the pots were drained and inoculated with a further 10 ml bacterial suspension. Three days later, they were again flooded with tap water for 24h. This flooding, draining and bacterial inoculation process was repeated until seedling evaluation. Seedlings were evaluated for root rot 12-15 days after inoculation with the fungal pathogen. the fresh weight, shoot height and number and root length were measured. Disease severity was estimated for each seedling using the following scale: 0 (no disease, roots free of symptoms), 1 (slight discolouration, water-soaked areas involving <10% of roots), 2 (water-soaked areas involving 10-20% of roots), 3 (20-50% roots water-soaked), 4 (water-soaked rot of >50% of the root system) (Falloon and Grogan, 1991). Pots were sown with five seeds, of which 3-4 germinated, with eight replicates of each treatment. Statistical data was analysed by ANVOA and Student-Newman Keuls (SNK) test for least significant difference.

4.4 RESULTS

4.5 Effect of *P.megasperma* on asparagus growth

The pathogen *P. megasperma* NZ006 was chosen for glasshouse trials of the biocontrol ability of PA147-2 and derivative strains. When seedlings were grown in the presence of the pathogen alone, 96.6% of plants developed root rot. *Phytophthora*-infected seedlings typically showed translucent and water logged roots, while uninfected roots appeared white and opaque (Fig 4.1) (Falloon and Grogan, 1991). *Phytophthora* infected seedlings were significantly reduced in shoot number, shoot height and root length. However, there was no significant difference in weight of diseased or healthy seedlings (Table 4.1).

4.6 Suppression of *Phytophthora* root rot by PA147-2

PA147-2 and derivative strains were tested for their ability to suppress the development of *Phytophthora* root rot of asparagus. Asparagus seedlings were inoculated with suspensions of either the wild-type PA147-2, Af^- mutant PA109, or Af^+ strain PA109R-186 (Table 2.1). Preliminary experiments showed inoculation of the seedlings with culture medium alone had no deleterious effect on seedling growth. Furthermore a control consisting of an overnight LB culture of DH5α(pUC18) had no effect on plant growth compared to the treatments with *Pseudomonas* strains (data not shown). PA109 was generated by Tn5 mutagenesis and is deficient for antibiotic production and fungal inhibition *in vitro*. PA109 was complemented by allele-replacement with a cosmid from a genomic library of the wild-type (PA109R-186). PA109R-186 was restored to the wild-type phenotype of antibiotic production and fungal inhibition *in vitro*, as described previously (Chapter 2). Asparagus seedlings were flooded and drained a number of times to favour establishment of the pathogen, and bacterial inoculations were repeated to

counter excessive diluting or washing of the bacteria from the soil. Counts Bacterial populations on the roots were not counted therefore no conclusion could be made from this study about colonisation ability of the mutant PA109 compared to the wildtype.

The roots were evaluated for the degree of rot using the disease scale described above (section 4.2.2). Seedlings grown in the presence of *P. megasperma* and PA147-2 showed significantly less infection (24%) and reduced disease severity compared with plants without bacterial treatment (Table 4.1). In contrast, 100% of seedlings treated with Af^- mutant PA109 developed root rot, indicating antibiotic production is necessary for biocontrol by PA147-2. Furthermore, there was a 23% reduction in *Phytophthora* infection on seedlings co-inoculated with PA109R-186 and the pathogen, confirming the importance of antibiotic production by this strain for biocontrol.

Treatment with either Af^+ or Af^- strains significantly reduced the number of diseased shoots. In the presence of the pathogen, shoot height of seedlings was reduced and treatment with Af^+ or Af^- bacterial strains did not increase shoot height (Table 4.1). Furthermore, in the absence of the pathogen, bacterial treatment of the seedlings also reduced the shoot height. This suggests that the negative effect of shoot height was an effect of PA147-2, rather than a consequence of *Phytophthora* disease. Root length was significantly reduced on seedlings treated with the pathogen alone. However, co-inoculation with the pathogen and any of the bacterial strains increased the average root length to that of the control. In the absence of the pathogen, root length was not significantly enhanced by bacterial treatment.

The average weight of the seedlings without bacterial treatment was not significantly affected by *Phytophthora* infection. However increases in plant weight of 40-60% in the presence of the pathogen, and 66-100% in the absence of

Figure 4.1

A Healthy uninfected roots of an asparagus seedling appear white and opaque compared to infected roots (B).

B *Phytophthora* infected roots, which are typically discoloured and translucent (arrow, blue appearance in photograph).

C *Phytophthora* infected seedling without bacterial treatment (left) and seedling co inoculated with the pathogen and strain PA147-2 (right).

Figure 4.1

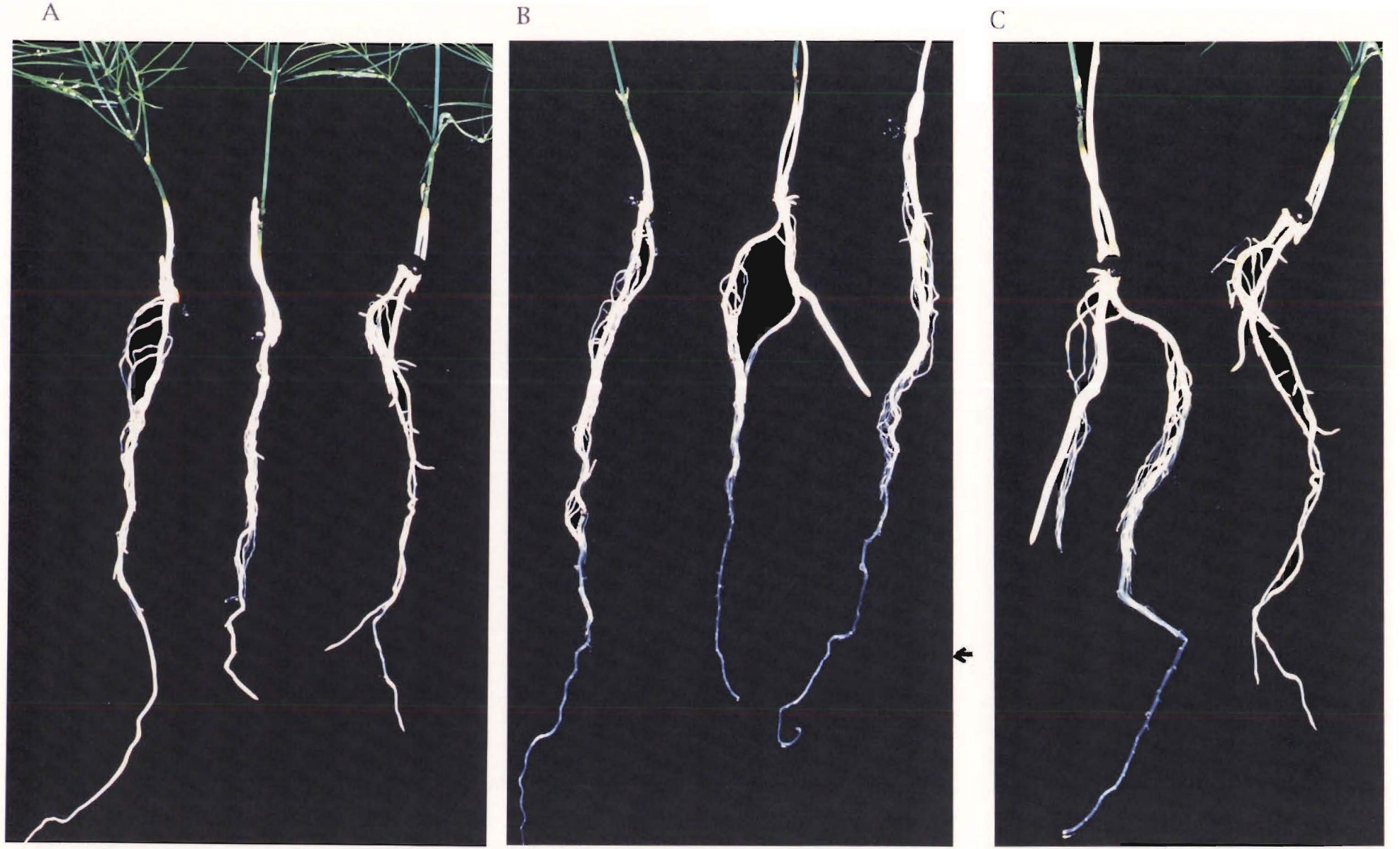


Table 4.1 Effect of antibiotic production on the suppression of *P. megasperma* NZ006 infected asparagus seedlings.

Treatment	Seedlings infected(%)[c]	Degree of rot[d]	Diseased shoots[e]	Root length(mm)[f]	Shoot height(mm)[g]	Plant weight(gm)[h]	Antifungal activity[i]
Control[z]	0	0	0	80.1 [b]	305.2 [a]	0.15 [a]	
NZ006 alone	96.6 [a]	3.37 [a]	0.77 [a]	64.3 [a]	242.6 [b]	0.15 [a]	
PA147-2 + NZ006	72.5 [b]	1.88 [b]	0.27 [b]	78.5 [b]	262.2 [b]	0.21 [b]	+
PA109R-186 + NZ006	73.3 [b]	1.41 [b]	0.13 [b]	88.5 [b]	267.8 [b]	0.25 [b]	+
PA109 + NZ006	100.0 [a]	2.22 [a]	0.12 [b]	72.7 [ab]	277.5 [b]	0.22 [b]	-
PA147-2				73.7 [b]	258.3 [b]	0.25 [b]	+
PA109R-186				74.0 [b]	262.3 [b]	0.30 [b]	+
PA109				77.6 [b]	261.3 [b]	0.30 [b]	-

[c] Values are the percentage of seedlings showing symptoms of *Phytophthora* NZ006 infection

[c-h] Values are the means of eight replications, each with 3-4 seedlings individually measured

[d] Average degree of rot observed based on 0-4 disease scoring scale of Falloon *et al.*, 1991

[e] Number of diseased shoots per seedling

[i] Inhibition of *Aphanomyces euteiches* ICMP 6478 and *Phytophthora megasperma* NZ006 on PBPDA

[z] No bacterial treatment or fungal pathogen NZ006 present

Values followed by the same letter are not significantly different (p=0.05) according to Student-Newman Keuls (SNK) test for least significant difference.

the pathogen were observed after co-inoculation with either Af^+ and Af^- strains (Table 4.1). The doubling in weight that was observed was most likely due to increase in the water content of the plants as shoot height, root length and shoot number were not affected. The measurement of fresh weights may not have given an accurate prepresentation of the weight gain due to bacterially-mediated growth enhancement. The application of the bacteria in LB media could account for some weight gain however a control of DH5α(pUC18) in LB broth was applied to plants with no affect on plant weight (data not shown). The results suggest that PA147-2 and derivative strains enhanced asparagus seedling growth in the absence of the pathogen. Furthermore, plant growth enhancement was independent of antibiotic production since it occurred with either Af^+ and Af^- strains.

4.7 DISCUSSION

The results of this study suggest a correlation between antibiotic production and fungal inhibition *in vitro* and disease suppression *in planta*. Seedlings treated with an antibiotic-deficient mutant were not protected from root rot. Restoration of antibiotic production to the Af$^-$ mutant by complementation was correlated with the ability to suppress disease *in planta,* thus supporting an important role for the antibiotic in disease control. It was necessary to add large volumes of bacterial suspension to the pots, to counteract the effects of repeated flooding and draining, required for uniform disease establishment. These high bacterial volumes would not be required in a field situation, as the same flooding and raining cycles would be less evident. In the field, asparagus plants generally recover from wet conditions during the drier summer, while the pathogen survives in the soil as spores, to infect again during the wet winter conditions (Falloon *et al.*, 1986). The pot assays therefore simulated these conditions, on a small scale. Future tests will involve assessment of PA147-2 biocontrol ability under field conditions. The reduction of disease severity from 97% to 72% reflects the potential of PA147-2 for biocontrol of asparagus root rot. Current control measures involve chemical sprays, as no *Phytophthora*-resistant cultivars are known (Falloon, 1991). The biological significance of this reduction and relevance to growers should be tested using field production of asparagus. The main consequence of *Phytophthora* infection is the reduction in quality and yield of spears (Falloon, 1982, Falloon *et al.,* 1986). Therefore, it is important to test these parameters such as the yield of marketable asparagus spears when the biocontrol ability of PA147-2 is assessed under field conditions. It can not be ruled out that PA109 has a reduced ability to colonise and establish populations on the roots compared to the wild-type, which could account for some of PA109's reduced biocontrol ability. Three previous studies found there to be no difference between the wild-type parental strains and the antibiotic-deficient mutants in their

colonisation ability (Bakker *et al.*, 1987, Loper 1988, Thomashow and Weller, 1988). The results presented in this chapter are consistent with recent studies of antibiotic production by *P. aureofaciens* Q2-87, a biocontrol agent of take-all of wheat (Vincent *et al.*, 1991) and *P. fluorescens* CHAO, which controls both take-all of wheat and black root rot of tobacco (Keel *et al.*, 1992). In these studies a positive correlation between antibiotic production and disease control is supported by the isolation of antibiotic-deficient mutants unable to suppress disease, and the subsequent restoration to wild-type activity *in vitro* and *in planta* by cosmid complementation. However, a mutant of strain CHAO, deficient for 2,4-diacetylphloroglucinol (Phl^-) and cyanide production (HCN^-), still showed some suppressive ability, suggesting that other mechanisms are involved, allowing a residual disease suppression *in vivo* (Keel *et al.*, 1992). In a recent study, two fluorescent pseudomonads were tested for their ability to control *Aphanomyces* root rot of peas and *Pythium* damping-off compared to standard commercial seed treatment with captan (Bowers and Parke, 1993). *P. cepacia* and *P. fluorescens* isolates significantly suppressed initial disease incidence by 59.3% and 31.2% respectively. Bacterial treatments were more successful at suppressing disease than captan, under dry soil conditions and may be an effective means of pathogen control (Bowers and Parke, 1994). A siderophore-deficient mutant of CHAO gave no evidence for a role of fluorescent siderophores in protection (Keel *et al.*, 1989), further indicating the involvement of multiple factors. Production of antibiotics such as oomycin A (Gutterson *et al.*, 1990) and pyoluteorin (Howell and Stipanovic, 1980) are implicated in the biocontrol of *Pythium* damping-off diseases. However, neither pyoluteorin, pyoverdine or the production of an uncharacterised antibiotic, accounted for the biocontrol activity of *P. fluorescens* Pf-5 against *Pythium* damping-off of cucumber, indicating that other mechanisms were also involved with this strain (Kraus and Loper, 1992).

In an earlier report, *in vitro* antibiosis correlated with growth promotion *in vivo* (Kloepper and Schroth, 1981) and none of the 16 antibiosis-negative mutants

caused an increase in plant growth compared to the wild-type parental strains. In this chapter there was an increase in the weight of asparagus seedlings both in the presence and absence of the pathogen. This suggests that growth promotion by PA147-2 is not related to the capacity to produce the antibiotic. This contrasts with a study of *Pseudomonas* strains applied to wheat seeds which suppressed the take-all pathogen, but did not promote growth other than by controlling disease (Weller and Cook, 1983). Three fluorescent pseudomonads which enhanced the growth and yield of winter wheat (de Freitas and Germida, 1990, 1991) also increased fresh weight of cabbage, lettuce and onion (Germida and de Freitas, 1994). However, the growth response of a *P. fluorescens* strain was eliminated when complete Hoagland solution was applied to plants. It is suggested that the increase in plant growth is a result of PGPR-enhanced root systems, which enable the plants to take up more water and nutrients. It is also possible that the application of nutrients favours the growth of other soil microbes, which out-compete the PGPR strains. PA147-2 may directly stimulate the root system of plants and improve the host's ability to uptake nutrients. Further characterisation is required to determine whether mechanisms other than antibiotic production result in a stimulatory effect on plant growth. Evaluation of PA147-2 disease suppression in the field will provide essential information on the efficacy of applying this strain as a biological control agent.

CHAPTER 5

Isolation and partial characterisation of an antifungal compound

Introduction

To date only a few antibiotics involved in biological control have been identified and most of these have been previously mentioned in this thesis. In summary, these include pyrrolnitrin and pyoluteorin, which are implicated in control of damping-off of cotton caused by *Rhizoctonia solani* and *Pythium ultimum* (Howell and Stipanovic, 1979, Kraus and Loper, 1989), of cucumber caused by *P. ultimum* (Kraus and Loper, 1992) and of radish caused by *R. solani* (Homma *et al.*, 1989); phenazine-1-carboxylic acid and its derivatives, active against *Gaeumannomyces graminis* var *tritici* (Brisbane and Rovira, 1988; Thomashow and Weller, 1988); oomycin A suppressive to damping-off of cotton caused by *P. ultimum* (Howie and Suslow, 1991); and 2,4-diacetylphloroglucinol, involved in control of black root rot of tobacco caused by *Thielaviopsis basicola* and take-all disease of wheat caused by *G. graminis* (Keel *et al.*, 1990a, 1992, Vincent *et al.*, 1991). This chapter reports the isolation of a compound from PA147-2 that inhibits *A. euteiches*. Three Af$^-$ mutants were also analysed to determine if Tn5 insertion resulted in loss of the same antibiotic. As only a small amount of inhibitory compound was obtained detailed analyses for its identification were not carried out. However HPLC analysis confirmed the mutants were unable to produce the antibiotic on PBPDA that is responsible for *A. euteiches* inhibition.

5.1 MATERIALS AND METHODS

5.2 Isolation of an antibiotic compound

PBPDA plates were inoculated with each *Pseudomonas* strain and incubated for 7 days at 25°C. The agar was homogenised in a blender and extracted with 3 volumes of 80% acetone. The agar-acetone slurry was agitated overnight at room temperature then filtered through cheesecloth and centrifuged at 9,000 rpm in a Sorvall JA14 rotor for 20 min at 4°C. Acetone was removed by rotary evaporation and NaCl (5.0g/100ml) dissolved in the remaining aqueous extract. The aqueous phase was then extracted three times with diethyl ether, and the ether fractions pooled and evaporated to dryness. The resulting residue was dissolved in 5ml acetonitrile. Antifungal activity in the crude extract was determined by bioassay against *A. euteiches.*

5.3 HPLC analysis of antifungal extracts

Using the retention properties of the antibiotic on silica, an extraction method was developed to reduce the background level of media constituents from PBPDA. Crude extracts were prepared as described above, however after acetone removal, the aqueous extract was applied to a C8 column (Analytichem Bond Elut; 500 mg/6.0 ml, sorbent mass/column volume). C8 columns were conditioned with consecutive washes of 100% methanol, 50% methanol and distilled H_2O prior to application of the aqueous extract. Antifungal compound was then eluted from the column with methanol and evaporated to dryness. The residue was dissolved in 1-2 ml distilled H_2O and 50-200ml injected onto high-pressure-liquid-chromatography (HPLC) system, using a 0.1% trifluoroacetic acid-acetonitrile gradient (30-60% over 20 mins). Samples were collected at a flow rate of 1.5ml/min and detected at a wavelength of 220nm. Fractions were evaporated to dryness and dissolved in DMSO

or acetonitrile for bioassay against *A. euteiches*, using the method described in Chapter 2.

5.4 RESULTS AND DISCUSSION

Isolation of an inhibitory compound

A crude extract with antifungal activity was first isolated from PA147-2 in a preliminary experiment to: a) determine if an antifungal compound could be detected and isolated, and b) design an appropriate protocol for a scaled-up extraction from PA147-2 and three Af$^-$ mutants grown under identical conditions. Inhibitory activity could not be detected in broth cultures, therefore the antibiotic was isolated from PA147-2 grown on solid media. In a preliminary experiment, a crude extract isolated from PA147-2 grown on PBPDA inhibited *A. euteiches*, and an active methanol fraction was isolated after partial purification on C8 columns. These extraction conditions were then applied to the analysis of Af$^-$ mutants PA1, PA109, PA138 and the wild-type PA147-2.

An HPLC-purified extract inhibited fungi *in vitro*

Prior to HPLC analysis, the crude and partially purified extracts were tested for inhibitory activity of fungal growth. Each fraction recovered from HPLC was bioassayed for activity against *A. euteiches*. One HPLC fraction, eluted at 18 minutes, inhibited *A. euteiches* on PBPDA (Fig 5.1 A). The presence of antifungal activity from PA147-2 corresponded to a peak on the HPLC profile (Fig 5.2 A). To confirm that the Tn5 insertions in PA1, PA109 and PA138 result in loss of the same antifungal activity, crude extracts were also prepared from these strains and analysed as described for PA147-2. No antifungal activity was detected in these extracts on a pre-HPLC bioassay (Fig 5.1 B). Furthermore, HPLC profiles of each

Figure 5.1

A: Assay for antifungal activity in eluted fractions after HPLC. Inhibitory activity was observed at 18 mins from PA147-2

B: Assay for activity in extracts from three Af^- mutants prior to HPLC

Figure 5.1

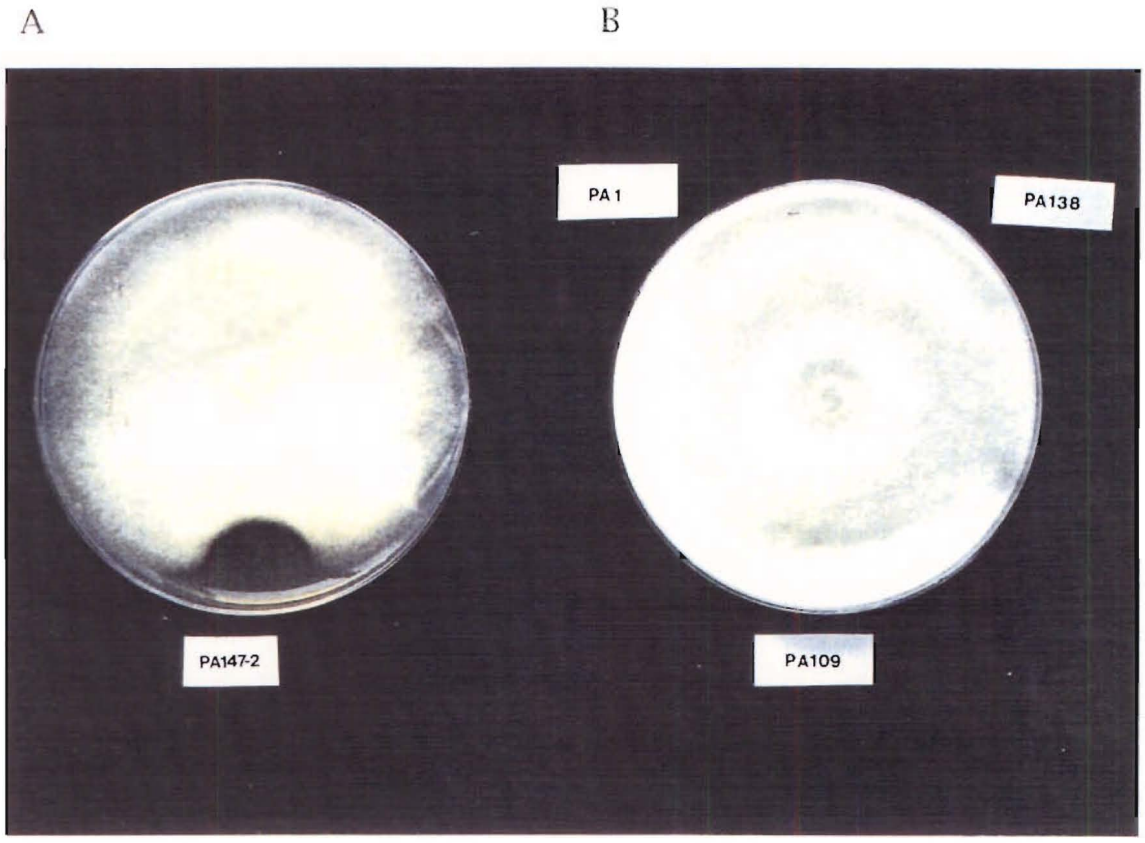


Figure 5.2

HPLC profiles of partially purified extracts from;

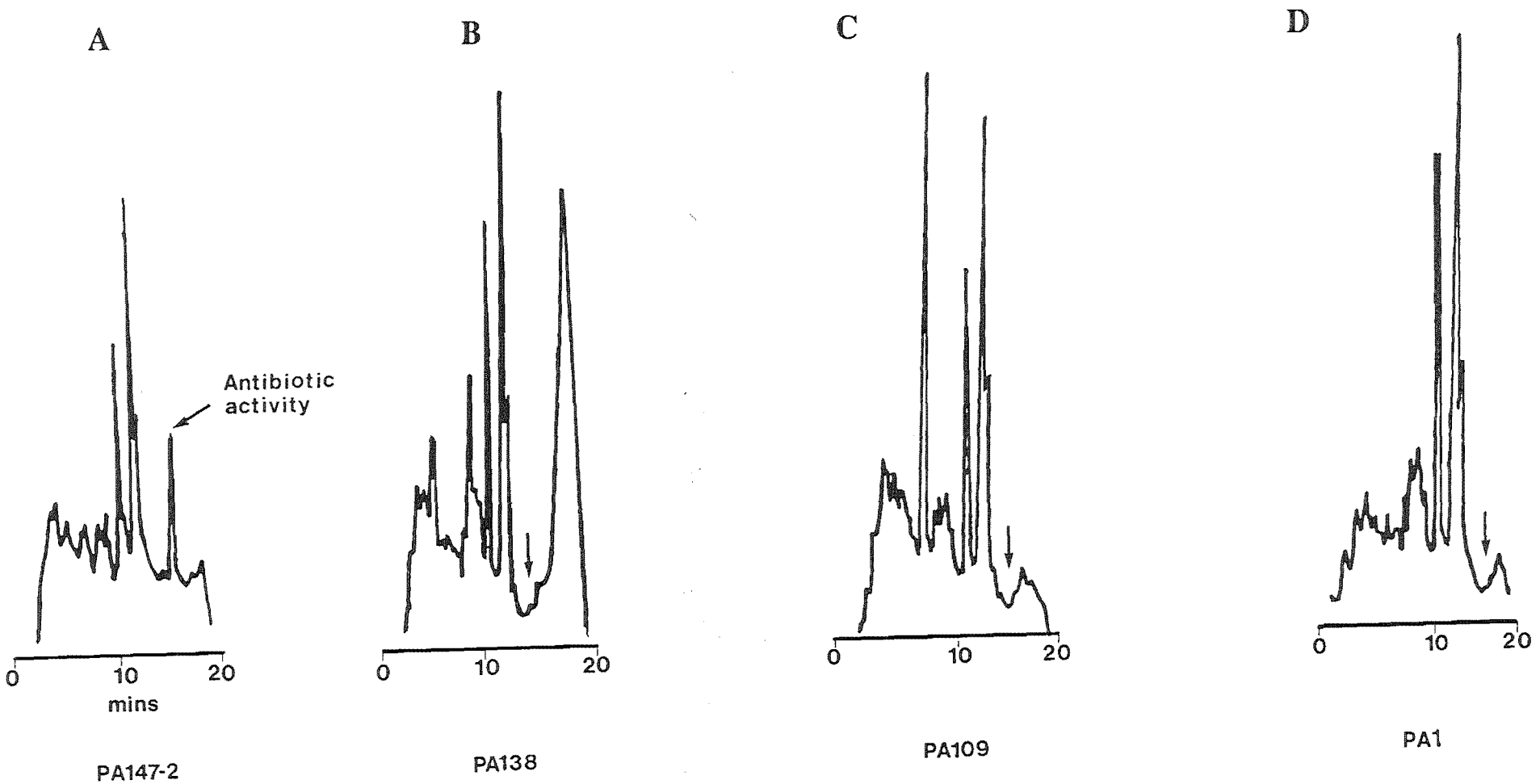
A PA147-2

B PA138

C PA109

D PA1.

An arrow indicates antibiotic activity in the fraction eluted at 18 mins from PA147-2 (A). The absence of an HPLC peak from the mutant extracts at 18 mins is shown with an arrow in B, C and D.


A
B
C
D
Antibiotic activity
0
10
20
mins
PA147-2
PA138
PA109
PA1

mutant extract show that the peak corresponding to antifungal activity in PA147-2 is absent in all three mutants (Fig 5.2 B, C and D). This result is similar to other studies which have used mutants for the characterisation of antibiotics: using HPLC, production of 2,4-diacetylphloroglucinol (2,4-DAPG) was detected in culture broths of two biocontrol pseudomonads, but not in the culture broths of Tn5 mutants of these strains (Vincent *et al.,* 1991, Shanahan *et al.,* 1992).

Tn5 has inserted in the same 16-kb *Eco*RI fragment in PA1 and PA109, and in an 8.2-kb *Eco*RI fragment in PA138. While it is not yet certain whether these *Eco*R I fragments are closely linked or scattered throughout the chromosome, it is clear that mutations in at least two different loci affect production of the same antibiotic compound (Chapter 2). The presence of different peaks in the HPLC profiles for the three mutants may be due to the accumulation of intermediates in the antibiotic biosynthetic pathway (Fig 5.2). Using these extraction conditions other Af^-, Tn5 mutants of PA147-2 could also be tested for loss of the antibiotic produced on PBPDA. To identify the antibiotic produced by PA147-2 it would be important to establish specific and defined growth conditions that are optimal for antibiotic production. For example, an antibiotic was isolated from *P. aureofaciens* Q2-87 grown in chemically defined medium and identified as 1,3,6-trihydroxy-2,4-diacetylphenone (Harrison *et al.,* 1993).

Under these conditions, it was not possible to isolate sufficient quantities of the compound for nuclear magnetic resonance (NMR) and mass spectrophotometry analysis. The low yield may have been due to the nature of a large scale extraction and the loss of significant amounts of the compound while handling large volumes of organic solvents. Further refining of the extraction and elution process may help to increase recovery of this antibiotic. Furthermore, it is possible that the compound can be complexed by constituents in the media which would also reduce yield. A recent study showed that the amount of DAPG produced

by a biocontrol fluorescent pseudomonad was affected by higher temperature and certain carbon sources (Shanahan *et al.*, 1992). Optimum production of the antibiotic occurred at 12°C and when the surface area:volume ratio was increased, and was influenced by availability of sucrose, fructose or mannitol (Shanahan *et al.*, 1992).

A mutant phenotype such as loss of antibiotic production may also be affected by media composition. In this study, 13 Af^- mutants of PA147-2 produced inhibitory compounds against *A. euteiches* on media 523, defined by Kado and Heskett (1970), and on media chemically defined for *A. euteiches* growth (Yang and Schoulties, 1972), while remaining Af^- on PBPDA. The Tn5 mutants also inhibited *A. euteiches* on media 523 amended with 100mm $FeCl_3$, indicating siderophore production could not account for the observed inhibition. Pfender *et al.*, (1993) reported a similar observation where a mutant of biocontrol strain *P. fluorescens* Pf-5, that does not produce pyrrolnitrin, still shows inhibitory activity on media 523, suggesting that some biocontrol strains produce multiple inhibitory compounds in response to different nutritional conditions.

It seems likely that PA147-2 produces other antibiotics that remain to be detected. The conditions used to extract and purify an inhibitory compound from solid media may have excluded the isolation of other antibiotics. The compound produced by PA147-2 remains to be characterised biochemically, however, a molecular approach could be used to identify this antibiotic and determine whether it is a novel compound. The structural genes for biosynthesis of phenazine (Pierson III and Thomashow, 1992), oomycin (Gutterson, 1990) and 2,4-diacetylphloroglucinol (Vincent *et al.*, 1991) have been cloned and could be used to probe genomic DNA from Af^- mutants of PA147-2. This approach would initially indicate whether PA147-2 contains the structural genes for these antibiotics and

whether the Tn5 insertions in any of the Af$^-$ mutants correspond to these antibiotic biosynthetic genes.

CHAPTER 6

DNA Sequence Analysis

Introduction

An understanding of the regulatory elements and environmental factors that influence disease suppression is critical if biological control strains are to have a practical application in the field. The mutagenesis approach is a powerful tool to identify lesions in genes which may regulate the transcription of multiple biocontrol factors. Af- mutant PA109 is of particular interest as it identifies a genomic region of at least 13-kb that is required for inhibitory activity. The success of identifying genes involved in antifungal activity through DNA sequencing and homology searches is entirely dependent on the extent of sequence information available in the databases.

Two-component regulators and biological control

Adaptive response systems in prokaryotes often involve signal-transduction proteins, known as two-component regulatory proteins. A comparison of a variety of adaptive response systems (Ronson *et al.*, 1987, Stock *et al.*, 1989), showed each component has a number of highly conserved amino acids and defined biochemical properties. One family of proteins, classified as sensory or histidine protein kinases (HPK), contains a conserved histidine residue which is phosphorylated and four additional blocks of conserved amino acids. The other protein component of this system is the response regulator (RR), which is the substrate for the histidine kinase. All response regulators contain a domain of at least two aspartate residues and a conserved lysine residue (Alex and Simon, 1994). Signal transduction occurs when the sensor protein (HPK) detects a signal molecule which results in auto phosphorylation at the conserved histidine

residue. A phosphoryl group is then transferred to the response regulator, which in turn, activates transcription of a number of genes (Kofoid and Parkinson, 1992; Parkinson, 1993). In some systems, both the kinase and the response regions are present on the same protein and these are termed "hybrid kinases" while in other systems the kinase activity is regulated by way of protein-protein interactions by a separate component (Alex and Simon, 1994). Adaptive-response systems have been identified in more than 10 different prokaryotic species and are involved in more than 20 different systems including osmoregulation, chemotaxis, nitrogen and phosphate regulation and pathogenicity (Nixon *et al.,* 1986, Bourret *et al.,* 1991, Stock *et al.,* 1989 and references therein).

Recent studies have identified two component regulatory genes that are involved in the expression of genes for biological control. *P. fluorescens* strain BL915 produces several biologically active compounds and protects cotton seedlings from *R. solani* (Lam *et al.,* 1994, Hill *et al.,* 1994). A number of pleiotropic mutants were isolated with deficiencies for the production of pyrrolnitrin, chitinase and cyanide. Genetic complementation studies showed two genes were required for the coordinated expression of multiple antifungal metabolites in strain BL915. One of these regulatory genes shares homology with a transcriptional activator, *gacA*, as well as other known gene activators. The second gene shares homology with a bacterial sensor, *lemA,* which encodes a histidine kinase protein. In the phytopathogen *P. syringae*, this protein controls lesion formation as well as syringomycin and protease production (Willis *et al.,* 1990, Hrabak and Willis, 1992, 1993).

The proposed counterpart of *lemA*, the response regulator, was first described in *P. fluorescens* CHAO as the *gacA* gene (global regulator of antibiotic and cyanide) (Laville *et al.,* 1992). Derivatives of CHAO with a *gacA* mutation no longer produce pyoluteorin, DAPG or cyanide. The GacA protein (24-kDa) is a response regulator

of the FixJ/DegU family of two component regulatory systems. A number of other studies have reported the isolation of global regulators involved in coordinated expression of biological control genes (Schnider *et al.* 1994, Lam *et al.*, 1994). From recent studies, it is clear that a single transposon insertion in a regulator element such as *gacA/lemA* may have a profound affect on several phenotypes, such as production of multiple antibiotics. Six mutants of *P. fluorescens* Pf-5, each with a single Tn5 insertion, did not produce pyoluteorin, pyrrolnitrin or cyanide (Kraus and Loper, 1992). In these three **a**ntibiotic **p**roduction (*apd*) mutants Tn5 had inserted within 1.5-kb of each other. The amino acid sequence indicates the *apd* region is closely related to the *lemA* gene (Loper *et al.*, 1994) and suggests that the *apd* region is a global regulator of at least two antibiotics and cyanide.

In this chapter, the defects in three of the five Af$^-$ mutants sequenced were identified through database searches. The mutation in PA109 was shown to be in a gene encoding a two-component regulatory protein, indicating that Tn5 had inserted in a regulatory region involved in antibiotic production. The identification of PA109 as a regulatory gene with similarity to other two-component regulators in bacteria, led to further sequencing of the wild-type region. A 2.0-kb wild-type fragment corresponding to the Tn5 mutation in PA109 was subcloned and sequenced in two orientations using ExoIII nuclease-digested deletions. The contribution of this result to the understanding of biological control by PA147-2, and to the growing evidence for global regulators in disease suppression is also discussed.

6.1 MATERIALS AND METHODS

6.2 DNA sequencing of five Tn5-containing clones

Five pBR322-based clones pFC1, pFC109, pFC30, pFC35 and pFC26 were chosen for sequence analysis. pFC1 and pFC109 were chosen as they contained Tn5 insertions in the same 16kb genomic fragment. Clones pFC30 and pFC35 were chosen on the basis that PA30 and PA35 were auxotrophic, while pFC26 was chosen as Tn5 had inserted into a small *Eco*RI fragment, approximately 5kb. Each clone contains a *Eco*RI::Tn5 insert cloned from the respective parental mutant. The primer chosen was a 17mer from the end of the right inverted repeat of Tn5. Normally the primer would bind to the almost identical left and right inverted repeats, therefore in order to prime and sequence from only one side of Tn5, a strategy of deletion cloning was used. This strategy and the maps of the resultant clones is shown in Fig 6.1.

6.3 Sequence analysis of a regulatory region

To sequence pSEQ1, ten deletions generated by ExoIII digestion (Nested Deletions Kit, Pharmacia), were chosen to cover the entire 2.0-kb fragment (5 for each strand and orientation)(Fig 6.3). These were sequenced using an Applied Biosystems 373A DNA Sequencing System. A database search was carried out with the DNA and predicted protein sequence using FASTA and BLAST database search programs. The sequence analysis program DNA Strider™, SeqEd™ and MacMolly™ (Apple Macintosh) were used for comparative alignments, overlaps and protein translation of DNA sequences.

6.4 DNA manipulations

Plasmid DNA for cloning procedures was prepared by standard methods (Sambrook *et al.,* 1989). Ligations were carried out according to the manufacturer's instructions (Bethesda Research Laboratory, NEB); however for deletion cloning, ligation reactions were carried out in 40-50 ml volumes. Double stranded plasmid DNA was prepared by standard methods and purified by caesium chloride gradient. Routinely, 2-5 mg of plasmid DNA was used for sequencing reactions. Plasmid denaturation, primer annealing, labelling reactions and gel electrophoresis were carried out according to the manufacturer's instructions (Sequenase, USB).

6.5 Sequence analysis

DNA sequences were analysed using Genbank and the protein translation programs FASTA and BLASTX (Gish and States, 1993).

6.6 RESULTS

6.7 Deletion cloning strategy for sequencing five cloned mutants

Five cloned mutants were digested with a restriction enzyme that digests the vector and Tn5 but not the insert. Tn5 contains two *Hind*III sites and a single *Bam*HI site. All clones without a *Hind*III site (box 1, 3, 4 and 5) in the insert were digested with *Hind*III and religated in dilute ligation conditions to favour recircularisation. The *Hind*III site in pBR322 and one of the *Hind*III sites in Tn5 ligated to form a deletion clone lacking the kanamycin resistance gene encoded by Tn5. All deletion clones were resistant to ampicillin and sensitive to kanamycin and tetracycline. In pFC35 (box 2) *Bam*HI replaced *Hind*III to generate a deletion clone. Each deletion clone contains only one side of Tn5. A 17mer primer was designed from the end of the right inverted repeat (AAACGGGAAAGGTTCCG) (Auerswald *et al.*, 1981) to sequence from the end of Tn5 and into the flanking insert DNA.

6.8 Sequence analysis of five Tn5 clones

The genbank database was searched with the DNA and predicted protein sequence from the five deletion clones. The clones pFC1 and pFC26 showed no significant homology with known genes in the Genebank database. Protein sequences of pFC30 and pFC35 showed significant homology with the *aroB* gene in *E. coli* (pFC30, 96%) and the *carA* gene in *E. coli* (pFC35, 71%) and *P. aeruginosa* (79%) (Fig 6.2). The protein sequence of pFC109 identified a regulatory protein that has similarity to *lemA*, a member of the family of regulatory elements known as two-component regulatory proteins (Hrabak and Willis, 1992; Stock *et al.*, 1989) (Fig 6.2).

Figure 6.1

Deletion cloning strategy for DNA sequencing of five cloned Tn5 mutants.

Boxes 1-5 show the enzyme digestion of each original Tn5 clone (plain text) and the resulting deletion clone underneath (bold text). The arrow above the deletion clones denotes priming from one side of Tn5, and the direction of sequencing into the flanking insert DNA.

B = *Bam*HI H = *Hind*III S = *Sac*I RI = *Eco*RI. The black box in each original clone represents pBR322.

Deletion cloning strategy for double stranded sequencing

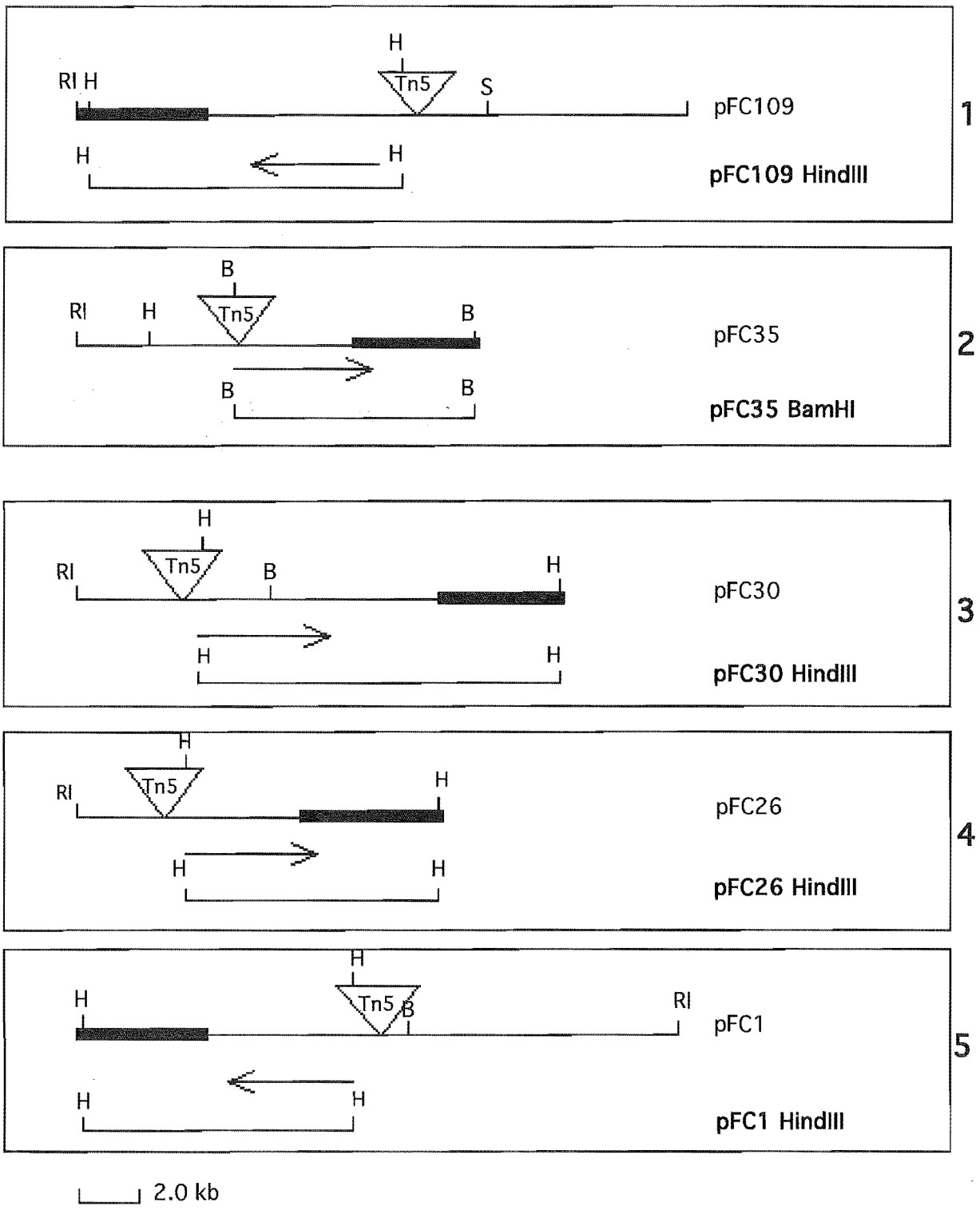


Figure 6.2

Sequence homology for mutants PA30, PA35 and PA109

Amino acids which are conservative replacements (Dayhoff, 1969) are connected by dotted lines and identical amino acids are connected by solid lines.

A: Sequence homology for pFC30

```
pFC30       F I Q V P T T L L S Q V D S S V G G K T G I N H P L A R T
            | | | | | | | | | | | | | | | | | | | | : | | | | | | |
E.coli      F I Q V P T T L L S Q V D S S V G G K T A V N H P L A R T
                130                 140                 150
```

96% identity with *E. coli* 3-Dehydroquinate synthase (*aroB*) in 29 amino acid overlap

B: Sequence homology for pFC35

```
pFC35       T M S L S D Y L K A N N T V A I A G I D T R R L T R L L
            |   : | | : | | | : : | : | | | | : | | | | : | | | | |
E. coli     T E D L S S Y L K R H N I V A I A D I D T R K L T R L L
              : |   | | | | : : : : | | | | : | | | | : | | | : |
P.aeruginosa K Q S L P S Y L K E N D T V A I A G I D T R R L T R I L
```

79% homology to *P. aeruginosa* carbamoyl synthase (*carA*) (Wong and Abdelal, 1990)
89% identity to *E. coli* carbamoyl synthase (*carA*) (Piette *et al.*, 1984)

C: Sequence homology for mutant pFC109

```
pFC109      Q I L V N L I G N A L K V H R A G H L H R R T P L A D P
            | | |   | | :   | | : |     | : |   :     |     | : | :
LemA        Q I L T N L V S N A I K F T R E G T I V A R A M L E D E

pFC109      G P R I A V V H C T V R D R G I G S A E A R L M F D - -
              :     |   :     : |   |   | | |   :       :
LemA        T E E H A Q L R I S V Q D T G I G L S S Q D V R A L F Q

pFC109      A F Q Q A D S S I S R R T Y Y T G L G L P I A417
            | | : | | |   | : | |         | | | | |   | :
LemA        A F S Q A D N S L S R Q P G G T G L G L V I S494
```

61% identity with *P. syringae* Lesion Manifestation (*lemA*) (Hrabak and Willis, 1992).

6.9 Identification of PA30 as *aroB*

The *aro*B gene encodes the enzyme 3-dehydroquinate synthase and catalyses the conversion of 3-Deoxy-*D*-arabino-heptulosonate 7-phosphate to 3-dehydroquinate in the shikimic acid pathway (Pittard, 1987) (Fig 6.2A, Appendix 1). A mutation in *aroB* prevents the biosynthesis of the aromatic amino acids phenylalanine, tryptophan and tyrosine. Auxotrophic mutant PA30 was unable to grow on minimal media unless all three aromatic amino acids were supplied (data not shown). Thus, PA30 does not inhibit *A. euteiches* on PBPDA due to Tn5 mutation of the *aroB* gene, which may be involved in supplying precursors for antibiotic biosynthesis. The elucidation of the biochemical pathways for synthesis of antifungal antibiotics is still largely unexplored. Phenazines are synthesised via the shikimic acid pathway, with chorismate as the branch point intermediate (Turner and Messenger, 1986). It is possible the antibiotic produced by PA147-2 on PBPDA also involves chorismate. PA30 did not inhibit *A. euteiches* on PBPDA supplemented with phenylalanine, tryptophan and tyrosine (data not shown), however, this would be expected if the antibiotic branch point lies earlier in the shikimic acid pathway, as in the case of phenazines.

Attempts to determine the antibiotic branch point using PA30 were not conclusive: an exogenous supply of chorismate, shikimate or both compounds did not restore PA30 to fungal inhibition on PBPDA (data not shown). This may be due to the inability of PA30 to take up these compounds and utilise either or both for antibiotic biosynthesis. The efficiency with which such intermediary metabolites gain entry to the cell varies with bacterial species (Pittard, 1987). Phosphorylated intermediates cannot enter the cell and dephosphorylated derivatives are generally not true intermediates (Pittard, 1987). Shikimate is one exception to this rule, while chorismate does not act as a growth factor to relieve blocked pathways in many cases, probably due to the failure of cell uptake. Tryptophan was recently

shown to be a precursor of pyrrolnitrin biosynthesis (Zhou *et al.*, 1992), an antibiotic produced by several biocontrol pseudomonads (Homma, 1994), thus it is possible that PA147-2 may also produce this antibiotic.

6.10 Identification of PA35 as *carA*

The region mutated by Tn5 in PA35 shows 71% and 79% homology to the *carA* gene from *E. coli* and *P. aeruginosa* respectively (Fig 6.2B, Appendix 1). The *carA* gene encodes the small subunit of the enzyme carbamoylphosphate synthetase, and has been cloned and characterised in *E. coli* (Piette *et al.*, 1984, Glandsorff, 1987), *S. typhimurium* (Kilstrup *et al.*, 1988) and *P. aeruginosa* (Wong and Abdelal, 1990). In *P. aeruginosa*, carbamoylphosphate synthetase (CPSase) is required for the synthesis of carbamoylphosphate which is a intermediate in the biosynthesis of arginine and pyrimidines, as well as for the utilisation of arginine via the arginine deaminase pathway (Abdelal *et al.*, 1982). It is uncertain how this enzyme may be involved in antibiotic production by PA147-2. Supplementing PBPDA with arginine and uracil did not result in antibiotic production by PA35, however, if the precursor(s) for antibiotic production are synthesised early in the arginine/uracil pathway, exogenous supply of these amino acids would be expected to have no effect. PA35 however, only grew on minimal media when it was supplemented with both arginine and uracil and did not grow when either compound was supplied individually. This is consistent with the predicted identity of this gene.

6.11 PA109 has homology to the regulator *lemA*

In PA109, Tn5 has inserted in a region with significant homology to the histidine protein kinase region or "sensor" of several bacterial signal-transduction proteins, known as two-component regulators (Ronson *et al.*, 1987). The predicted

amino acid sequence for 721 nucleotides flanking the Tn5 insertion in PA109, was 67% homologous to the sensor histidine kinases *BarA* (**b**acterial **a**daptive **r**esponses, Nagasawa *et al.*, 1992) and *RpfC* (Regulation of Pathogenicity Factors, Tang *et al.*, 1991) proteins, and 61% homology to the *lemA* histidine kinase region (**l**esion **m**anifestation, Hrabak and Willis, 1992) (Fig 6.2C). The exact site of Tn5 insertion was not identified as sequences close to the Tn5 primer could not be obtained with manual sequencing. The 2.0-kb wild-type fragment homologous to this region was subcloned from cosmid pPS7138 for further DNA sequencing (Fig 6.3).

6.12 Cloning of a regulatory region for DNA sequencing

To sequence the regulatory region identified in PA109, the complementing wild-type region was subcloned (pSEQ1). A 4-kb *Bgl*II-*Sac*I wild-type fragment was subcloned from the cosmid pPS7138 into pUC18 (*Bam*HI/*Sac*I), to construct pSEC1(DH5α). A 2.0kb *Eco*RI/*Bam*HI fragment from pSEQ1 was then cloned into the vectors pBluescript II KS+ and pUC18 (Yanisch-Perron *et al.* 1985) for DNA sequencing in both orientations (Fig 6.3).

6.13 DNA Sequence analysis of a putative regulatory region

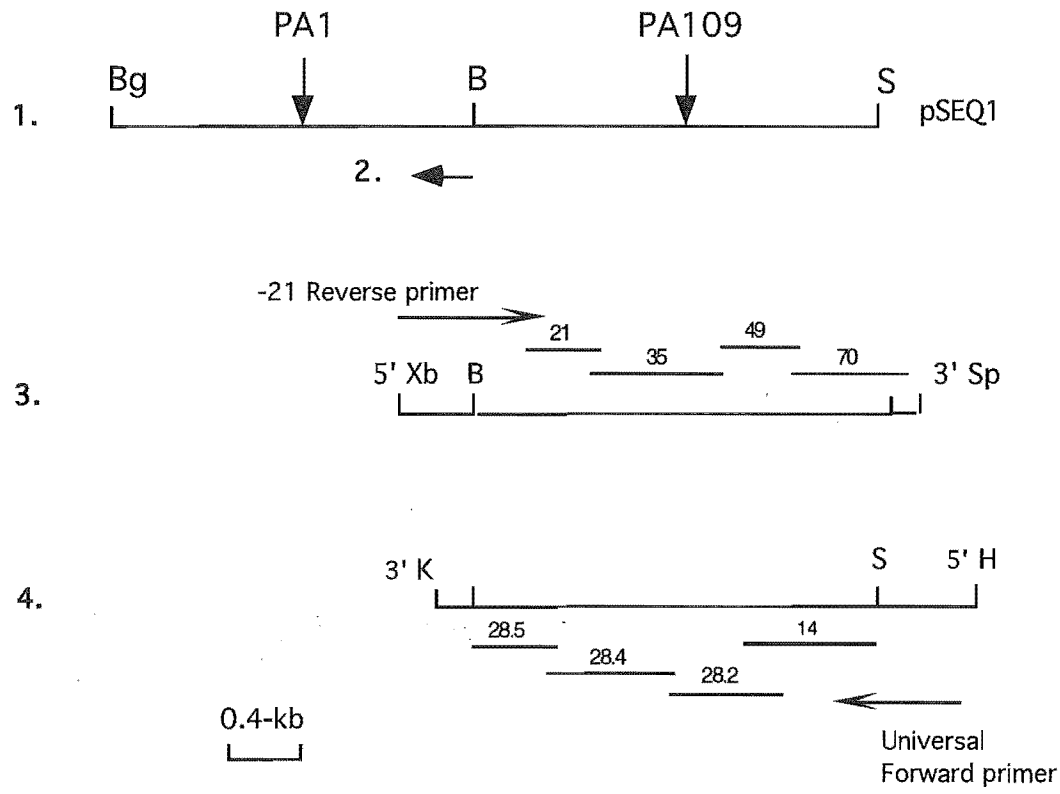
A 2,050 bp *Bam*HI-*Sac*I DNA fragment was sequenced on both strands and analysed with a universal codon usage (Fig 6.4). A database search identified a possible open reading frame (ORF) with a putative start codon at 316 nucleotides and stop codon at 1,993 nucleotides. This open reading frame was designated **a**nti**fu**ngal activity (AfuA), and showed homology to segments of LemA (62%) (Hrabak and Willis, 1992) and BarA (67%) (Nagasawa *et al.*, 1992). However, the absence of promoter recognition sequences (Helman and Chamberlain, 1987) in the HPK region suggests that an alternative start codon may exist beyond the *Bam*HI site.

The ORF in frame 1 was chosen from the computer alignment programme, as it contains a single start and stop codon in this frame compared to multiple stop codons present in all other frames. Comparisons of AfuA with other two-component regulators indicated that all the individual residues that are conserved in most HPK-RR proteins, are also present in the proposed protein sequence for AfuA (Fig 6.5). However there is also significant homology with these proteins 5' to the selected AfuA start codon at 315 amino acids, further suggesting that the correct ATG for *afuA* lies outside the cloned fragment. Therefore further DNA sequencing beyond the *Bam*HI site is required to obtain the entire *afuA* gene. Amino acids 1 to 424 of AfuA, contain a putative histidine kinase (HPK) region and a Kyte-Doolittle hydrophobicity plot showed a hydrophobic region between amino acids 10 and 70 which might suggest the presence of a transmembrane domain in this protein (Figure 6.6). Transmembrane domains have been observed in a number of HPK proteins (Stock, 1990, Nifja, 1991). However, some other two-component regulators such as *FrzE* (McBride *et al.*, 1989), and *DegS* (Henner *et al.*, 1988) lack discernible membrane-spanning sequences (Stock et al., 1989, references therein, Alex and Simon, 1994). There is major hydrophilic region in the *afuA* gene between amino acids 70-125, which may correspond to a periplasmic domain involved in ligand sensing, also a feature of hybrid kinases (Swanson and Simon, 1994). A variation in the number of transmembrane domains is typical of hybrid kinases (Swanson and Simon, 1994). The presence of both bydrophobic and hydrophilic regions from amino acid 150 to the end of the protein sugests portions of the AfuA maybe associated with the membrane or the cytoplasm (Figure 6.6). Further DNA sequencing past the *Bam*HI cloning site will help to define the likely start codon and membrane spanning region of this protein more accurately. The (G + C) content of the *afu*A gene was 58%, slightly below the average (G + C) content of 60-62% in the *P. fluorescens/P. putida* group (Palleroni, 1984), but identical to that of the *gac*A gene from *P. fluorescens* CHAO (Laville *et al.*, 1992).

A response regulator (RR) region was identified within amino acids 480 to 653, of the same ORF containing the HPK (Fig 6.4). The presence of both a sensor kinase and a response regulator in the same ORF suggests that AfuA is a hybrid kinase (Swanson and Simon, 1994). The *AfuA* ORF of 1,614 nucleotides could code for a protein as large as 538 amino acids with a predicted molecular weight of 59-KDa. The Tn5 insertion in PA109 is approximately 1-kb from the *Bam*HI cloning site (estimated from restriction mapping of pFC109) and is located in the HPK portion of AfuA. The exact position of Tn5 insertion in the HPK region remains to be determined by generating primers to sequence from the flanking DNA towards the junction sites of Tn5 insertion.

Figure 6.3

Cloning strategy for sequencing a regulatory region in PA147-2



1. A 4.0-kb *Bgl* II-*Sac* I wild-type fragment was cloned from the cosmid pPS7138 into pBR322, and designated pSEQ1. The sites of Tn5 insertion in the corresponding genomic region of Af$^-$ mutants PA1 and PA109 are shown for reference

2. A 2.1-kb *Bam*HI-*Sac*I fragment was cloned into pUC18. The clone was linearised with *Xba*I and *Spe*I providing a 5' ExoIII nuclease sensitive overhang for digestion, and 3' protected overhang respectively

3 The same fragment was also cloned into pBluescript KS+ and linearised with *Hind*III and *Kpn*I to provide a 5' ExoIII-sensitive and 3' ExoIII-protected overhang, respectively. Primers show the direction of sequencing. Numbered lines above each clone indicate the approximate location of the overlapping deletion clones, chosen for sequencing. B = *Bam*HI, Sp = *Spe*I, Xb = *Xba*I, H = *Hind*III, S = *Sac*I, K = *Kpn*I

Figure 6.4

Nucleotide and predicted amino acid sequence of AfuA

(*Bam*HI)

```
Q  D  L  R  Q  E  L  D  H  T  G  Q  L  I  A  N  Q  L  A  P  A 21
CAGGACCTGCGCCAGGAACTGGACCACACCGGCCAGTTGATCGCCAACCAACTGGCGCCGGCC
                                                              63
T  E  Y  G  V  I  S  G  N  N  D  V  L  E  S  L  L  R  A  T  L 42
ACCGAATACGGGGTGATTTCCGGCAACAACGACGTGCTTGAAAGCCTGTTGCGGGCCACCCTC
                                                             126
A  T  P  H  V  R  F  L  E  I  Q  D  S  A  E  N  I  L  V  Y  V 63
GCCACGCCGCACGTCCGCTTCCTGGAGATTCAGGACAGCGCAGAAAATATCCTGGTGTATGTC
                                                             189
E  Q  P  S  E  K  H  D  R  S  L  S  V  K  F  S  R  R  R  F  A 84
GAGCAACCGTCGGAGAAGCACGATCGCTCGCTGTCGGTGAAATTTTCCAGGCGCCGATTCGCC
                                                             252
C  N  I  S  Q  L  G  D  T  N  R  R  L  T  K  R  R  C  P  S  W 105
TGCAACATATCCCAGCTGGGCGATACCAACAGGCGATTGACCAAACGCCGATGCCCATCGTGG
*                                                            315
M  N  S  E  L  G  D  L  S  R  H  I  N  N  L  A  D  G  L  N  Q 126
ATGAACTCGGAGCTGGGCGACCTGTCGCGCCACATCAATAACCTCGCCGATGGCTTGAACCAG
                                                             378
A  S  R  E  Q  H  Q  A  M  A  Q  L  I  Q  T  R  E  E  A  E  R 147
GCCAGTCGTGAACAGCACCAGGCCATGGCCCAGTTGATCCAGACCCGCGAAGAAGCCGAGCGG
                                                             441
A  N  N  A  K  S  D  F  L  A  M  M  S  H  E  I  A  Q  P  M  N 168
GCGAACAATGCCAAGTCGGACTTTCTGGCAATGATGAGCCATGAAATTGCGCAGCCGATGAAC
                                                             504
G  V  L  G  M  L  Q  L  L  E  T  T  V  M  T  E  E  Q  T  E  Y 189
GGGGTGCTGGGCATGCTGCAACTGCTGGAAACCACCGTCATGACCGAGGAACAGACCGAATAC
                                                             567
A  A  L  A  S  E  S  T  E  H  L  L  K  S  D  Q  R  H  L  D  F 210
GCGGCGCTGGCCTCGGAGTCCACCGAACACCTGCTGAAGAGTGATCAACGACATCTCGATTTT
                                                             630
C  A  S  S  G  R  P  W  S  L  A  H  P  F  D  L  V  G  L  F  G 231
TGTGCATCGAGCGGGCGGCCCTGGAGCTTAGCACATCCGTTCGACCTGGTGGGGTTGTTCGGA
                                                             693
F  R  P  G  F  R  R  R  Q  Q  G  G  W  V  L  M  P  S  P  G  L 252
TTTCGCCCAGGCTTCAGAAGGCGCCAACAAGGGGGTTGGGTTCTAATGCCGTCCCCAGGCCTG
                                                             756
G  C  R  S  T  V  D  R  P  I  R  N  F  C  D  F  R  I  P  E  L 273
GGTTGTCGAAGTACAGTGGACCGACCGATTCGAAATTTTTGTGACTTTCGAATTCCTGAATTA
                                                             819
T  G  G  L  T  W  T  I  V  T  W  P  N  S  C  Y  C  T  R  L  V 394
ACGGGAGGACTTACCTGGACCATTGTAACTTGGCCAAATTCTTGTTACTGTACTCGGTTGGTT
                                                             882
L  N  S  R  A  E  Y  L  F  F  K  Q  F  L  K  L  E  R  Q  R  G 315
TTGAATTCCCGGGCTGAATATTTATTTTTCAAGCAATTTTTAAAGTTGGAACGTCAACGCGGG
                                                             945
L  A  L  E  V  P  I  P  Q  G  L  G  S  L  Q  V  Q  G  D  P  T 336
CTGGCGCTCGAAGTGCCGATCCCCCAGGGCCTGGGCTCGCTGCAGGTACAGGGCGACCCGACC
                                                            1008
R  I  R  Q  I  L  V  N  L  I  G  N  A  L  K  V  H  R  A  G  H 357
CGCATCCGGCAGATTTTGGTGAACCTGATCGGCAATGCCCTGAAAGTTCACCGAGCAGGGCAC
                                                            1071
```

```
L  H  R  R  T  P  L  A  D  P  G  P  R  I  A  V  V  H  C  T  V 378
CTTCACCGTCGAACCCCACTGGCAGACCCTGGACCACGAATTGCTGTGGTTCACTGCACCGTA
                                                              1134
R  D  R  G  I  G  I  S  A  E  R  L  E  L  M  F  D  A  F  Q  Q 399
CGCGATAGGGGAATTGGCATTTCCGCCGAGCGCCTGGAACTGATGTTCGATGCGTTCCAGCAA
                                                              1197
A  D  S  S  I  S  R  R  Y  G  G  T  G  L  G  L  P  I  A  R  T 420
GCCGACAGTTCCATTTCAAGACGTTACGGCGGCACCGGACTGGGACTGCCTATCGCACGAACC
                                                              1260
W  P  N  A  L  A  A  P  A  R  Q  S  E  E  G  H  G  S  V  F  T 441
TGGCCGAACGCATTGGCGGCACCTGCGCGCCAGAGCGAAGAAGGCCACGGTTCGGTGTTCACC
                                                              1323
F  L  K  A  C  M  P  L  A  I  W  Q  Q  S  L  P  V  L  A  P  N 462
TTTCTAAAAGCTTGCATGCCGTTGGCGATCTACCAGCAGAGCTTGCCGGTGCTCGCGCCGAAT
                                                              1386
T  E  G  N  G  R  A  G  E  G  R  N  V  L  L  V  E  D  N  P  V 483
ACCGAAGGCAATGGCCGTGCGGGTGAAGGGCGCAACGTGTTGCTGGTGGAAGACAACCCGGTC
                                                              1449
N  R  T  V  V  E  A  M  L  R  S  L  G  F  E  V  S  I  A  T  D 504
AATCGCACGGTGGTCGAAGCCATGCTGCGCAGCCTGGGGTTCGAGGTGAGCATCGCCACCGAC
                                                              1512
G  A  E  A  I  R  S  A  E  S  L  I  F  T  A  I  L  M  D  C  R 525
GGCGCCGAAGCGATTCGCAGCGCCGAGAGCCTGATTTTTACCGCAATCCTGATGGACTGCCGA
                                                              1575
L  P  G  I  D  G  Y  E  A  T  R  Q  I  R  Q  L  P  G  C  A  E 546
CTGCCGGGCATCGATGGCTACGAGGCTACCCGGCAGATTCGCCAGTTGCCCGGTTGCGCCGAG
                                                              1638
L  P  I  I  A  L  T  A  N  A  L  Q  G  D  R  E  A  C  L  A  A 567
CTGCCAATCATTGCCCTCACGGCCAATGCCTTGCAGGGCGATCGGGAAGCCTGCCTGGCAGCT
                                                              1764
G  M  N  D  Y  L  A  K  P  F  K  R  T  D  L  Q  Q  I  L  Q  R 588
GGAATGAACGATTACCTGGCAAAGCCGTTCAAACGCACGGATTTGCAGCAAATCCTGCAGAGA
                                                              1827
W  V  A  V  T  R  C  H  Q  P  T  A  T  G  V  K  D  E  S  A  A 609
TGGGTTGCAGTAACACGGTGCCATCAGCCAACTGCGACTGGCGTGAAAGACGAAAGTGCCGCA
                                                              1890
V  L  G  T  R  T  G  P  Q  T  G  P  V  Y  N  F  S  A  Q  V  Y 630
GTCTTAGGCACCCGAACGGGCCCGCAAACCGGCCCGGTTTATAATTTCAGTGCACAAGTGTAC
                                                              1953
I  H  D  L  D  A  V  T  F  T  T  T  Q                        643
ATTCATGACCTTGACGCTGTGACTTTCACTACAACGCAATAGTCTATGTGTAGGCTGCCGATA
                                                              2016
TGAGGCATGAACGCTTCAGTCGGCCGGGAAGATTTGCCCTGCCCTGCCGACATGGGATTGATT
                                                              2079
GAGGAG
      2085 (SacI)
```

The nucleotide and predicted amino acid sequence for the *AfuA* ORF presented in a 5' to 3' direction. Bars indicate the location of highly conserved residues, common to the HPK and RR region of two component regulators. An asterisk indicates a putative translation start codon.

Figure 6.5

Comparison of the derived amino acid sequence from AfuA with some proteins of two-component regulatory systems:

A: Alignment of the histidine protein kinase region of AfuA. In this region, the conserved histidine (H), asparagine (N) residues and conserved glycine-rich regions are boxed

B: Alignment of the response regulator region of AfuA. The conserved aspartic acid (D) and lysine (K) residues are boxed. In both **A** and **B**, amino acids which are conservative replacements (Dayhoff, 1969) are connected by dotted lines and identical amino acids are connected by solid lines. LemA (Hrabak and Willis, 1992), RpfC (Tang *et al.*, 1991) and BarA (Nagasawa *et al.*, 1992)

Figure 6.5: A

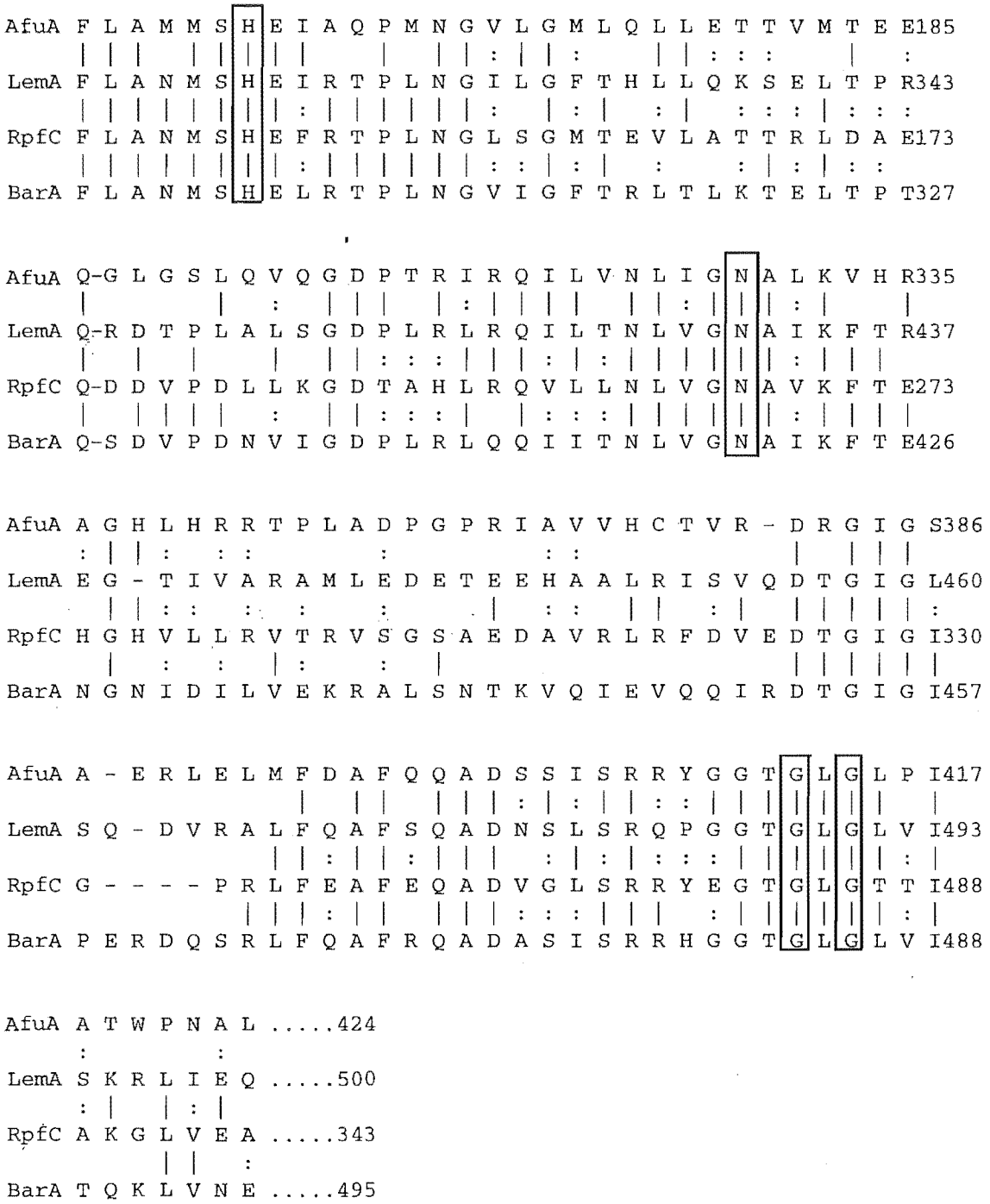

AfuA F L A M M S H E I A Q P M N G V L G M L Q L L E T T V M T E E185
LemA F L A N M S H E I R T P L N G I L G F T H L L Q K S E L T P R343
RpfC F L A N M S H E F R T P L N G L S G M T E V L A T T R L D A E173
BarA F L A N M S H E L R T P L N G V I G F T R L T L K T E L T P T327
AfuA Q-G L G S L Q V Q G D P T R I R Q I L V N L I G N A L K V H R335
LemA Q-R D T P L A L S G D P L R L R Q I L T N L V G N A I K F T R437
RpfC Q-D D V P D L L K G D T A H L R Q V L L N L V G N A V K F T E273
BarA Q-S D V P D N V I G D P L R L Q Q I I T N L V G N A I K F T E426
AfuA A G H L H R R T P L A D P G P R I A V V H C T V R - D R G I G S386
LemA E G - T I V A R A M L E D E T E E H A A L R I S V Q D T G I G L460
RpfC H G H V L L R V T R V S G S A E D A V R L R F D V E D T G I G I330
BarA N G N I D I L V E K R A L S N T K V Q I E V Q Q I R D T G I G I457
AfuA A - E R L E L M F D A F Q Q A D S S I S R R Y G G T G L G L P I417
LemA S Q - D V R A L F Q A F S Q A D N S L S R Q P G G T G L G L V I493
RpfC G - - - - P R L F E A F E Q A D V G L S R R Y E G T G L G T T I488
BarA P E R D Q S R L F Q A F R Q A D A S I S R R H G G T G L G L V I488
AfuA A T W P N A L 424
LemA S K R L I E Q 500
RpfC A K G L V E A 343
BarA T Q K L V N E 495

Figure 6.5: B

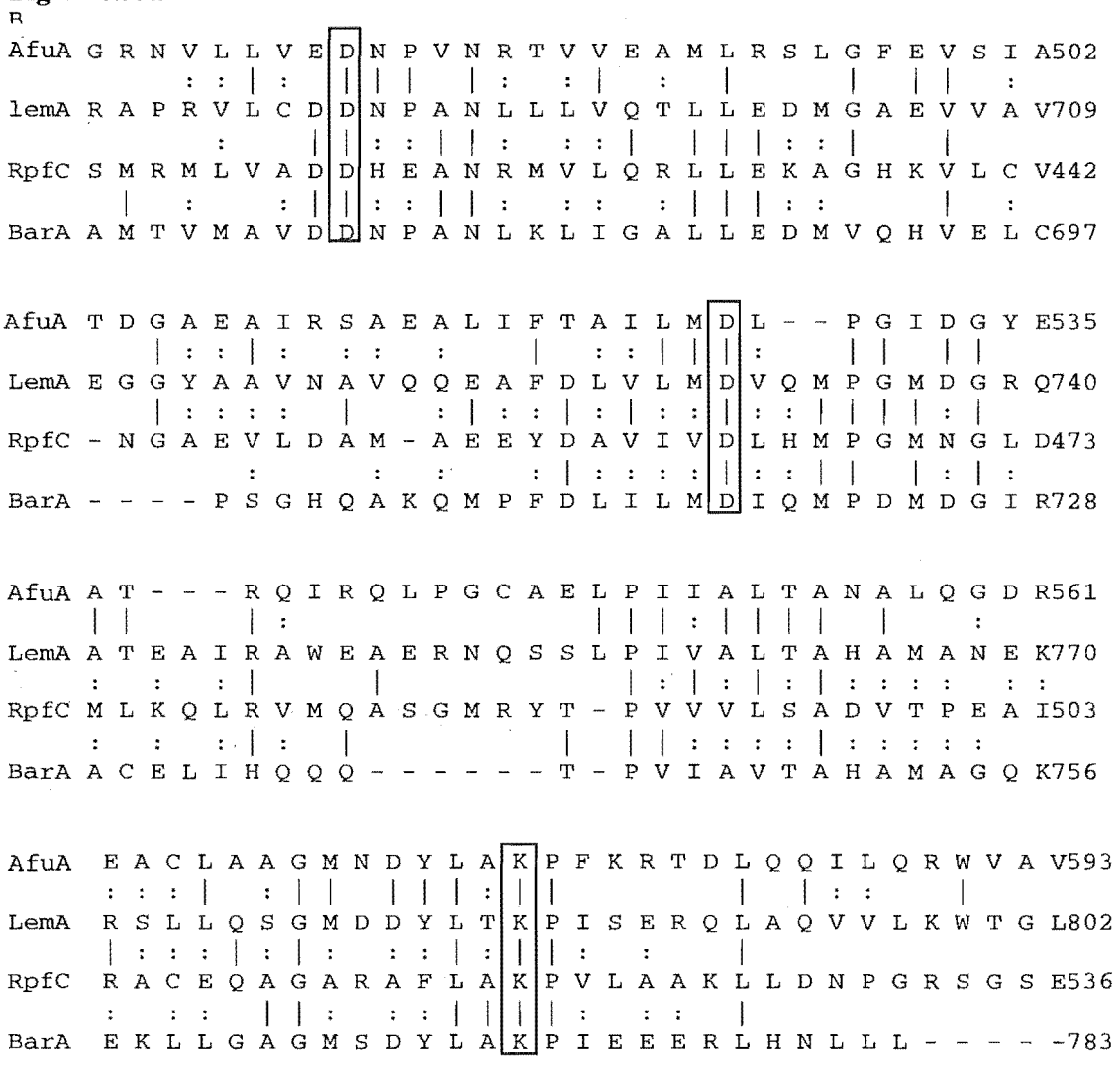
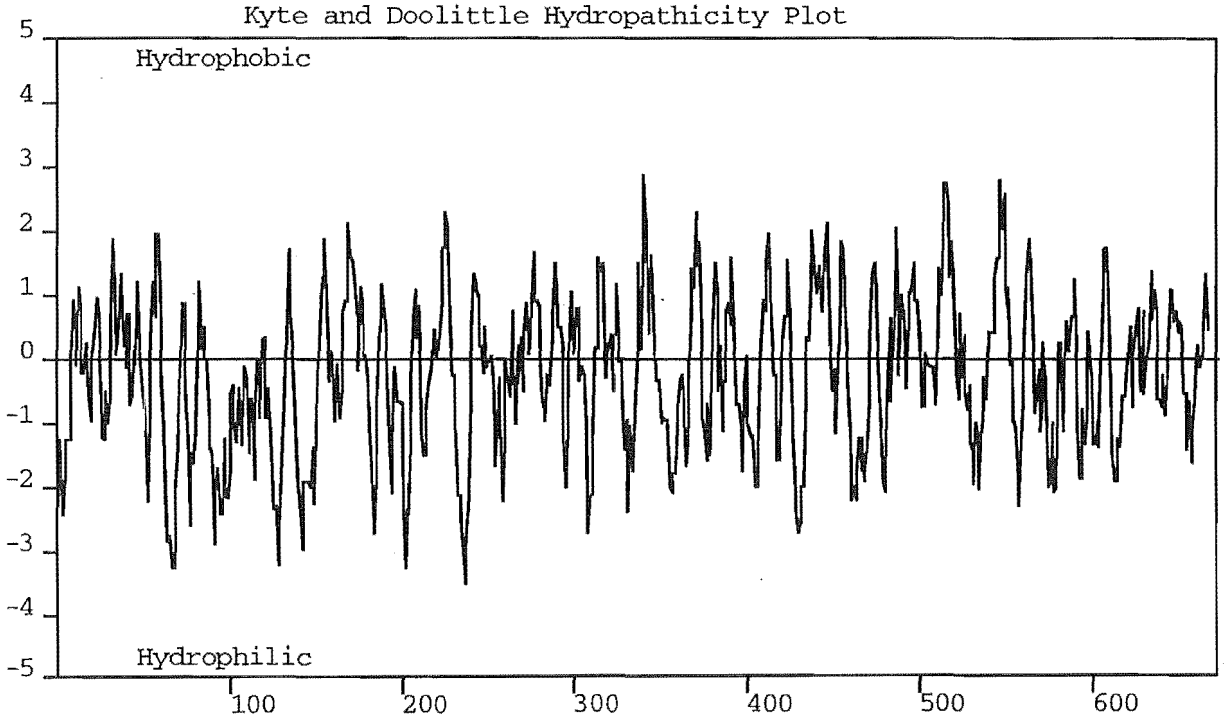

B
AfuA G R N V L L V E D N P V N R T V V E A M L R S L G F E V S I A502
lemA R A P R V L C D D N P A N L L L V Q T L L E D M G A E V V A V709
RpfC S M R M L V A D D H E A N R M V L Q R L L E K A G H K V L C V442
BarA A M T V M A V D D N P A N L K L I G A L L E D M V Q H V E L C697
AfuA T D G A E A I R S A E A L I F T A I L M D L - - P G I D G Y E535
LemA E G G Y A A V N A V Q Q E A F D L V L M D V Q M P G M D G R Q740
RpfC - N G A E V L D A M - A E E Y D A V I V D L H M P G M N G L D473
BarA - - - - P S G H Q A K Q M P F D L I L M D I Q M P D M D G I R728
AfuA A T - - - R Q I R Q L P G C A E L P I I A L T A N A L Q G D R561
LemA A T E A I R A W E A E R N Q S S L P I V A L T A H A M A N E K770
RpfC M L K Q L R V M Q A S G M R Y T - P V V V L S A D V T P E A I503
BarA A C E L I H Q Q Q - - - - - - T - P V I A V T A H A M A G Q K756
AfuA E A C L A A G M N D Y L A K P F K R T D L Q Q I L Q R W V A V593
LemA R S L L Q S G M D D Y L T K P I S E R Q L A Q V V L K W T G L802
RpfC R A C E Q A G A R A F L A K P V L A A K L L D N P G R S G S E536
BarA E K L L G A G M S D Y L A K P I E E E R L H N L L L - - - - -783

Figure 6.6

Hydrophobicity plot of the predicted AfuA protein



6.14 DISCUSSION

AfuA is a putative two-component regulator

A large open reading frame, designated AfuA, was identified by a database search to have significant homology with members of the family of sensor/regulator proteins in bacteria. PA109 has a Tn5 mutation in the sensor, HPK region of AfuA, which prevents production of the antibiotic on PBPDA (Chapter 2) and reduces the biocontrol ability of the wild-type on asparagus plants (Chapter 4). This genetic region is probably required to detect an environmental signal(s) which subsequently regulates antibiotic biosynthesis. Homology of the predicted AfuA protein to LemA (61%) and BarA (67%) is not high compared to other putative regulators of this type, showing homologies to LemA of greater than 80% (Stephen Lam, personal communication, CIBA-Geigy Corp., Research Triangle Park, North Carolina, USA). This suggests that AfuA may be a novel protein. Since a major hydrophobic region upstream of the HPK region was not found within the *Bam*HI-*Sac*I fragment, it is possible that the ORF continues past the *Bam*HI cloning site, into the adjacent fragment (Fig 6.3). Mutagenesis experiments (Chapter 3) indicate this adjacent region is part of a putative cluster, of at least 13-kb, involved in fungal inhibition. AfuA may be required for the regulation of adjacent or nearby antibiotic genes within this cluster. It has been shown in *Pseudomonas* sp., that transcription of genes for the biosynthesis of antifungal metabolites is controlled by one or more regulatory genes, that are either clustered with biosynthetic genes (Gutterson, 1990, Pierson III and Thomashow, 1992) or located distally in the genome (Laville *et al.,* 1992). The proposed AfuA protein contains both a sensory kinase and a response regulator domain in its primary amino acid sequence, a feature in common with several other sensor/regulator proteins: BarA (Nagasawa *et al.,* 1992), ArcB (Iuchi *et al.,* 1990), BvgC (Arico *et al.,* 1989), VirA (Leroux *et al.,* 1987), RpfC (Tang *et al.,* 1991) as well as LemA (Hrabak and Willis, 1992), termed 'hybrid kinases" (Alex and Simon, 1994;

Swanson and Simon, 1994) all contain the kinase and regulator domains in their primary sequence.

AfuA and global regulation

A single transposon insertion in a regulator element, such as *gacA/lemA*, may have a profound affect on several other phenotypes such as production of multiple antibiotics (Loper *et al.*, 1994, Lam *et al.*, 1994). Six mutants of *P. fluorescens* Pf-5, each with a single Tn5 insertion, did not produce pyoluteorin, pyrrolnitrin or cyanide (Kraus and Loper, 1992). In three of these antibiotic production mutants (*apd*), Tn5 had inserted within a 1.5-kb region. Sequence analysis indicates the *apd* region is closely related to the *lemA* gene (Loper *et al.*, 1994) and suggests that the *apd* region is a global regulator of at least two antibiotics and cyanide. The term "global regulator" cannot yet be assigned to AfuA, as so far only one major inhibitory compound has been observed, with a significant role in PA147-2 biological control. However, PA147-2 probably produces other antibiotics, since PA147-2 and some Af$^-$ mutants inhibit fungal pathogens on different growth media (Chapter 2). It is possible that AfuA is also involved in the production of other undetected antifungal metabolites or may only be involved in regulating the production of a single antibiotic.

To establish whether AfuA has a specific or global regulatory activity, the *afu*A gene could be introduced into strains which do not produce antifungal metabolites. Heterologous activation of latent genes by a global regulator was recently demonstrated (Lam *et al.*, 1994). A *gac*A homologue enabled *P. fluorescens* strain BL914 to produce pyrrolnitrin, chitinase and cyanide, even though this strain does not normally produce these compounds. It would also be interesting to examine whether GacA-like activators, identified in other *Pseudomonas* strains, could activate the production of pyrrolnitrin, pyoluteorin and 2,4diacetylphloroglucinol and cyanide in PA147-2.

AfuA and biological control

How AfuA functions to regulate antibiotic production remains to be determined. There is growing evidence that global regulators have an important role in biological control, and particularly in production of antibiotics. A cosmid from a *P. fluorescens* CHAO genomic library enhanced expression of pyoluteorin and 2,4-diacetylphloroglucinol (Maurhofer *et al.,* 1992). Antibiotic enhancement could be due to a gene dosage effect of the cosmid, causing an increase in the concentration of antibiotic precursors. However, recent results suggest that a 2.3-kb genomic region is involved in positive regulation of pyoluteorin and 2,4-diacetylphloroglucinol in this strain. In this case, the regulator is probably different from the GacA positive regulator already identified in CHAO, as a *gac*A-negative mutant could not be complemented by the 2.3-kb region (Schnider *et al.,* 1994).

The 13-kb cluster, which contains AfuA, may contain genes for biosynthesis of the antibiotic produced by PA147-2. The biosynthetic regions have been identified for oomycin A (Gutterson, 1990), phenazines (Pierson and Thomashow *et al.,* 1992), cyanide (Voisard *et al.*, 1989, Haas *et al.*, 1991), 2,4-diacetylphloroglucinol (Bangera *et al.,* 1993) and pyoluteorin (Loper *et al.,* 1994). The biosynthesis genes occur as relatively large contiguous DNA regions for oomycin A (15kb) (Gutterson, 1990), or pyoluteorin (21kb) (Loper *et al.,* 1994), which contrasts to the small size of non-linked regulatory regions such as GacA (0.64kb) (Laville *et al.,* 1992), or LemA (2.7kb) (Hrabak and Willis, 1992). Further sequencing of the cluster region and Southern hybridisation with known antibiotic biosynthetic genes will contribute to the characterisation of the PA147-2 antibiotic and its regulation.

FINAL DISCUSSION

PA147-2 produces an antibiotic involved in biocontrol

The findings presented in this study suggest a potential role for *P. aureofaciens* PA147-2 as a biological control agent, for suppression of disease caused by plant fungal pathogens and promotion of plant growth. A *P. aureofaciens* strain, PA147-2, was shown to produce an antibiotic on culture media (Af^+) that inhibited the growth of several fungal phytopathogens tested. Tn5 mutants (Af^-), deficient for antibiotic production, no longer inhibited the pathogens on the PBPDA medium. Complementation by allele-replacement, with cosmids from a genomic library of the wild-type, restored wild-type activity in two mutants. These mutants, PA1 and PA109, contain single Tn5 insertions separated by 2.1 kb, in the same genomic *Eco*RI fragment (Chapter 2). Further mutagenesis experiments using a miniTn10 transposon identified at least 13kb of genomic DNA that is involved in antibiotic-mediated biological control on PBPDA (Chapter 3). The results from both mutagenesis experiments suggests that the DNA region probably contains a cluster of genes for regulation of the antibiotic. This cluster may also contain genes for synthesis of the antibiotic.

The mutagenesis approach remains the primary method for understanding the contribution of various biocontrol mechanisms to disease suppression. Many of the studies cited in this thesis report the use of mutants to establish a correlation between fungal inhibition *in vitro* and disease suppression *in vivo*. In general, these studies establish that some metabolites contribute to but do not account for all of the biological control observed. The fact that in some cases, mutants continue to suppress disease *in vivo*, albeit at reduced levels, may be due to induction of the plant's natural defence mechanisms. Colonisation of wheat roots

by *P. fluorescens* CHAO was found to induce leaf resistance to tobacco necrosis virus (TNV) (Maurhofer *et al.,* 1994a). Siderophore production was involved in induction of leaf resistance to TNV, but had no role in disease suppression on the roots. Therefore, it is important to evaluate several mutants defective in production of a single antibiotic, to fully understand the contribution of an antibiotic. A single mutation may result in a range of pleiotropic effects causing other undetectable changes in phenotype from the parental strain.

PA147-2 reduces disease and stimulates growth of asparagus

The biocontrol ability of the wild-type PA147-2, PA109 and complemented mutant PA109R-186 was tested under growth room conditions. The results suggest that antibiotic production is an important mechanism for the protection of asparagus seedlings from *Phytophthora* root rot. There was a positive correlation between *in vitro* production of the antibiotic on PBPDA and biological control *in planta.* Plant growth was stimulated when asparagus seedlings were inoculated with either Af^+ or Af^- strains of PA147-2, suggesting that growth promotion is independent of pathogen suppression (Chapter 4). It is difficult to directly compare these results with other studies of biological control in glasshouse conditions as the bacterial strain, host plant, fungal pathogen, soil and growth conditions are all different from those used in other test systems. Furthermore, under field conditions PA147-2 may not show the same biological control ability or growth enhancement observed in the growth room assays. An antibiotic produced *in vitro* may not be important in the field because the strain uses aggressive colonisation and competitive exclusion on the root to control other microbes. The most effective biocontrol may yet be achieved through the application of mixed strains, with multiple mechanisms of disease suppression. This was observed with some fluorescent *Pseudomonas* strains that are capable of suppressing pathogens *in vivo* when combined, but unable to confer biocontrol individually (David Weller, personal communication, USDA, Agricultural Research Service, Pullman, USA).

PA147-2 produces an antifungal antibiotic

An unidentified antibiotic was isolated from solid media extracts of PA147-2. Three Af- mutants PA1, PA109 and PA138 no longer produce this antibiotic due to Tn5 insertion in genetic loci required for its production (Chapter 5). The absence of antibiotic production by PA109 supports the observation in Chapter 4 that PA109 does not protect asparagus seedlings from *Phytophthora* rot, presumably because it does not produce the antibiotic. DNA sequence analysis identified the mutation in PA109 as a two-component regulator (AfuA), necessary for antibiotic production in culture and protection of asparagus seedlings *in planta* (Chapter 6). The additional peak observed in the HPLC profile of mutant PA109 is interesting in the light of the identification of a regulatory gene. This could be explained if a compound that interacts specifically with this regulator has accumulated in the absence of functional regulator protein. Two other mutants, PA30 and PA35, are defective in amino acid biosynthetic pathways, which may provide essential precursors for antibiotic synthesis.

Regulation of biological control mechanisms

The discovery of global regulators in biological control strains is an exciting new direction of research. Based on its similarity to other global regulators, it is probable that AfuA regulates a number of different genes. To understand the regulatory processes it will be necessary to identify the environmental stimuli which signal gene expression. These stimuli are probably complex. For example, *gacA*-regulated antifungal metabolites are required for protection of tobacco roots against black root rot, but *gacA* is not involved in the induction of resistance in tobacco leaves (Maurhofer *et al.*, 1994a). The host plant has a major influence on the activity of *gacA* in bacteria, and this may be due to the varied and complex nature of root exudates. It will be useful to test PA147-2 and PA109 on different hosts to determine whether AfuA is influenced by production of root exudates

from various host plants. Root exudates are complex and contain many compounds that may specifically induce bacterial genes, without serving as a carbon or energy source. It is therefore likely that some of these compounds are directly involved in bacterial signal transduction (van Elsas and van Overbeek, 1994). GacA is involved in regulation of antibiotic and cyanide synthesis against *T. basicola* and *P. ultimum* on tobacco and cucumber respectively, but has no role in suppression of *G. graminis* and *P. ultimum* on wheat (Laville *et al.,* 1992). AfuA may not be involved in biocontrol by antibiotic production on different hosts and instead may regulate production of other antifungal metabolites whose activity is influenced by the host.

The research presented here provides a foundation for future studies involving *P. aureofaciens* PA147-2. Immediate future work could focus on the genes identified in the putative antifungal cluster. The use of reporter gene systems based on ice-nucleation (Lindgren *et al.*, 1989, Georgakopoulos *et al.*, 1994), β-galactosidase (Howie and Suslow, 1991) or bioluminesence (Shen *et al.,* 1992, Glover, 1994) would provide useful assessment of antibiotic production. The expression of genes under AfuA control could be monitored under different growth conditions such as soil type, temperature and on different host plants. Knowledge of the expression and regulation of these genes *in situ*, will provide opportunities for the improvement of bacterial biological control of fungal diseases of plants.

Appendix 1

Nucleotide sequence of pFC30 and pFC35

pFC30

5'TGTTCTTGCCAGCGGGTGGTTGATGCCGGTCTTGCCGCCCACCGACGAATCGACCTGGGACAGCAA
GGTGGTCGGTACCTGGATAAAGTCCAGGCGGCTGATAGCAGGCGCTGCAAAGCCCCCTGGGTGGCG
GTGTGCACGGCGACATGGCTGGCTTTGCAGCGCCTGCTATCAGCGCCTGGACTTTATCCAGGTACCGA
CCACCTTGCTGCAGGTCGATTCGTCGGTGGGCGGCAAGACCGGCATCAACCACCCGCTGGCAAGAAC
A3'

pFC35

5'ACACGATGTCCCTGTCCGATTACCTGAAAGCCAACAACGTTGTGGCGATCGCGGTATCGATACGC
GTCGCCTGACACGCATCCTGCGGTAAAAAGGCTCGCAGAACGGCTGCATCATGGCCGGTGACAACA
TCTTCCGAAGAAGCAGCAGTCGCAGCGGCCCAAGGTTTCCCTGGCCTGAAACGATGGATCCTGGCG
AAAGTCGTCAGCACCAATTAACGTACAGT3'

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